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I, LEANNE MYNOTT, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 8394 for a patent by THE AUSTRALIAN NATIONAL UNIVERSITY filed on 29 January 1999.

WITNESS my hand this
First day of March 2000

LEANNE MYNOTT
TEAM LEADER EXAMINATION
SUPPORT AND SALES

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The Australian National University

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A method for controlling plant pathogens, and agents useful for same"

The invention is described in the following statement:

A METHOD FOR CONTROLLING PLANT PATHOGENS, AND AGENTS USEFUL FOR SAME

FIELD OF THE INVENTION

The present invention relates generally to species and sub-species of the genus *Pseudomonas* which are capable of metabolizing D-glucose and other carbon sources to a corresponding sugar acid such as but not limited to gluconic acid, malic acid, glutaric acid, glutamic acid, glucaronic acid and galactonic acid. The level of production of the sugar acid is such that its concentration is sufficient to inhibit, retard or otherwise control the growth and/or viability of prokaryotic and eukaryotic organisms and, in particular, fungi and bacteria which infect or otherwise infest plants or parts of plants. The present invention further contemplates genetic material from the species or sub-species of *Pseudomonas* which encodes or otherwise facilitates synthesis of enzymes in metabolic pathways for sugar acid production from D-glucose or other carbon sources when said genetic material is introduced into other organisms or plants. The present invention further contemplates transgenic plants and microorganisms which have been genetically engineered to produce sugar acids at levels sufficient to control the growth and/or viability of pathogens. The present invention further provides compositions comprising sugar acids useful in controlling prokaryotic or eukaryotic growth and/or viability.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications are referred to by author in this specification are collected at the end of the description.

The increasing sophistication of recombinant DNA technology is greatly facilitating research and development in the agricultural and horticultural industries. However, despite advances in genetically manipulating plants including crops, microbial and insect infestation still accounts as a major cause of crop failure. There is a need, therefore, to identify and understand mechanisms of preventing microbial and insect infestation and to exploit these mechanisms in rendering plants pest resistant.

Interactions between microorganisms and plants do not necessarily result in damage to the plant. In fact, a large number of interactions between microorganisms and plants are mutually beneficial. The majority of these interactions are below ground in the soil between the microbe and plant roots. There are a few exceptions such as *Rhizobium* stem nodulation (nitrogen-fixing symbiosis) of the tropical legume *Sesbania rostrate* (Dreyfus and Dommergues, 1981) . Nitrogen fixation is of significant interest as it is conservatively estimated to be responsible for incorporating 122 million tonnes of nitrogen per annum. This is approximately 80% of all nitrogen that is added to the Earth's surface. The other 20% is mainly due to the application of chemical fertilizers (Gallon and Chaplin, 1987). However, agricultural chemicals have serious environmental implications and, hence, natural mechanisms, such as plant-microbe interactions, are now being considered.

Rhizobium root nodule symbiosis is by far the best understood beneficial plant-microbe interaction (Sprent, 1989) and specific genes involved in inducing specific responses on the root have been identified and studied in a prokaryotic system. There are a large number of more subtle plant-microbe interactions which do not induce such specific responses in the plant but nevertheless provide a benefit to the plant host and the microbe involved. For example associative nitrogen fixation (Berkum and Bohool, 1980) or disease protection (Cook and Baker, 1984) . This type of associative interaction is probably the most frequent plant-microbe interaction which occurs in nature. Therefore, it is important to understand the genetic nature of these interactions. Furthermore, interactions of plants with higher organisms such as fungi in mycorrhizal association (of fungi with roots) seem to be widespread in nature (including all families of flowering plants, some pteridophytes and bryophytes) and is of significant biological and economic importance (Richards, 1987) . However, due to their complexity, progress in understanding the molecular nature of these interactions has been slow.

Biological control protection refers to the introduction of microorganisms to control plant pathogens. This is a subtle plant-microbe symbiotic interaction which does not induce specific responses on the host plant but does provide significant benefits to the plant (Baker and Cook, 1974). There are a large number of systems in which biocontrol is effective (Weller, 1988). In

general, biocontrol agents have been shown to have a beneficial effect to the plant under controlled conditions in the glasshouse and in a large number of cases in the field (Baker and Cook, 1974) . As in *Rhizobium* nitrogen fixing symbiosis, the benefit to the biocontrol bacteria can only be surmised as being a safe haven from competition from other bacteria. There are currently numerous biocontrol agents used by farmers for disease control (Schroth and Hancock, 1981) , showing that it is effective and viable as a method to control plant diseases in the field.

Furthermore from the wide range of biocontrol protection demonstrated with different plant diseases (Baker and Cook, 1974) it has the potential to be used widely in agriculture. The two properties which make biocontrol agents preferable is that they are natural inhabitants and, as such, are less likely to have a detrimental effect on the environment. Secondly, they are relatively inexpensive and are an inexhaustible source, since microorganisms can rapidly and relatively easily be grown up in large amounts.

The most simple form of biological control is to stop disease on a plant by occupying the site of infection of a pathogen. For example the presence of a passive *Agrobacterium radiobacter* species on a plant sensitive to crown gall disease stops *Agrobacterium tumefaciens* (the causal agent) from causing crown gall (Kerr, 1972) . Although bacteriocin production (Kerr, 1980) may play a minor role in protection, it has been shown that the major reason is that the strain present on the plant is established and as such can out compete the pathogenic strain (Schroth and Hancock, 1981) . Therefore, the pathogenic strain is unable to establish itself and cause disease. The term "rhizobacteria" has been coined for a wide range of bacteria on different plant hosts which can aggressively colonise roots (Schroth and Hancock, 1982). This colonisation can significantly improve plant growth and yield. It is postulated that the presence of the rhizobacteria on the roots is a barrier which stops colonisation of any potential deleterious microorganisms can cause disease (Schroth and Hancock, 1982) . This can be effective in biocontrol such as in potatoes (Geels *et al.*, 1986) . These forms of biocontrol are used effectively in the field but very little is know about the exact process that leads to protection. Also in some cases, increased plant vigor could be due to additional undetected factors such as nitrogen fixation or antibiotic production (antibiosis).

The universal feature of all biological control bacteria in suppressing plant root diseases is their capacity to rapidly multiply in the rhizosphere of the root and to form an association with it (Suslow, 1982) . This process has been termed colonisation. This could range from bacteria being on or near the surface of the root, to very loose root attachment, to actually living inside the epidermal layer of the outer root surface (Costerton, 1984). The different colonisation host range shown by biocontrol bacteria (Weller, 1988) suggests their is host specificity in colonisation. Attempts to study root colonisation have given inconclusive results (Ligion *et al.* 1988) . Screening of over 1000 transposon mutants in biological control bacteria for the fungus take-all gave 65% mutants which had a reduced ability to colonize. Screening a number of these independent mutants showed all had a reduced growth rate. This suggested that a slower growth rate involving subtle metabolic changes led to poorer colonisation ability. In their study, Ligion *et al.* were unable to detect any colonisation deficient mutants which were not due to growth rate differences, so specific factors determining colonisation could not be identified. It is clear from such studies that a large number of fundamental processes which contribute to the make-up of the bacteria determine it's colonisation ability on the plant root. Since a large number of unknown processes are involved in colonisation, this could explain why storage and sub-culture of bacteria in the laboratory leads to loss of ecological competency in the soil (Weller, 1988) . Other specific factors thought to contribute to colonisation are: exopolysaccharides (EPS); chemotaxis; cell surface moieties determining physical attachment to the root (Weller, 1988). However, there have been no convincing data to show them being directly involved in biological control.

Biological control of soil borne pathogens has been extensively studied (Weller, 1988). The important bacterial factors identified where are responsible for their suppressive nature include association with the root ("colonisation"), production of antibiotics (defined as compounds which are active at a low concentration / ug) and which are toxic and inhibit the fungal pathogen and fluorescent siderophore production (high affinity iron, transport agents; Weller, 1988) . As there is host specificity in root colonisation (Suslow, 1982) , host plant factors may be responsible for the ability of the bacteria to colonise the roots. However, little is known of the plant 's role in this symbiosis.

There is host specificity in whether an antibiotic is effective against a pathogen and this might explain why some strains are ineffective against certain pathogens (Schroth and Hancock, 1981).

Also in some biological control protection, antibiosis has shown not to be involved (Kraus and Loper, 1992). There are other factors such as HCN production which have been shown to be involved in a small number of cases of biological control (Fravel, 1988).

In conclusion, a number of different mechanisms are operating in biological control protection of bacteria against pathogens. Significant factors important in biological control (such as colonisation, antibiosis, siderophore production etc.) have been identified. However, the influence of these factors varies with environmental conditions so it is difficult to assign a universal role for these factors in biological control (Weller, 1988).

The take-all disease caused by the fungus *Gaeumannomyces graminis* var *tritici* (or commonly known as the "take-all" fungus) is the most significant root disease of wheat around the world and currently leads to 10% loss of the annual wheat crop in Australia (Murray and Brown, 1987). Wheat yield increases of 30% have been demonstrated by biological control in field trials (Weller and Cook, 1983). Control methods have been effective for this disease. Rotational cropping with legumes grown between cereals or fallowing with good grass control has been shown to reduce the severity of the disease. Although fungicides have been tried they are expensive for broad acre farming and have generally been found to be not as effective in dry-land conditions where most wheat is grown.

Some *Pseudomonas* biological control bacteria have been identified which produce low molecular weight siderophore (sometimes fluorescent) compounds (high affinity iron transport agents) which complex with iron and are then actively transported inside the cell (Buyer and Leong, 1986). It is considered that part of the ability to protect against take-all is due to the siderophores efficiently chelating the iron in the root environment. The iron deficiency in the soil leads to a lack of an essential nutrient required for growth of the take-all fungus and thus limits the growth of the pathogen in the root environment (Leong, 1986). The molecular mechanisms involved in siderophore transport are well understood (Crosa, 1989). A decrease in protection by bacterial mutants which are deficient in siderophore production suggests that they may play a role in biocontrol. Siderophore production is thought only to be important in biological control protection (if at all) in neutral or alkaline soils which have low iron concentration. Iron

can be a limiting nutrient in these soils (Kloepper *et al.*, 1980). It was originally suggested that fluorescent siderophores produced by some of the *Pseudomonas* biological control bacteria may play an important role in suppression in alkaline soils. However, work by Hamdan *et al.* (1991) suggested that fluorescent siderophores play little or no role in suppression of the take-all fungus and the dominant important factor in disease suppression is anti-fungal agent production. Evidence now suggests that siderophore production is not an important factor and if it plays any role in take-all biological control it is only in alkaline soils.

Anti-fungal agents are generally considered to be organic compounds of low molecular weight produced by microorganisms which at low concentrations are deleterious to the growth or metabolic activities of other microbes (Fravel, 1988). Different antibiotic compounds are produced by different biocontrol bacteria and the same bacteria can produce multiple antibiotics.

Antibiosis deficient mutants are significantly reduced or lose their suppression of the take-all fungal plant pathogen in biological control (Fravel, 1988). It have demonstrated that suppression of take-all is directly related to the presence of the antibiotic in the root rhizosphere in the field (Thomashow *et al.*, 1990).

The nature of the anti-fungal compound produced by these biological control bacteria has been determined. For the control of the take-all pathogen only two compounds identified so far have been found to be effective. The first is phenazine -1-carboxylic acid which is a novel compound in bacteria is produced as a non-essential secondary metabolite (Thomashow *et al.*, 1993). The second compound identified to be inhibitory to the take-all pathogen is 2,4-diacetyl phloroglucinol, a normal intermediary in a pathway in bacteria (Keel *et al.*, 1992). This inhibits a range of fungal pathogens and is found in a wide range of Pseudomonads (Keel *et al.*,1996). However, in biological control bacteria, it is considered to be produced in a high amount, compared to what is produced in other organisms.

Phenazine has been extensively characterised in take-all biological control protection. The effectiveness of 2,4-diacetylphloroglucinol against take-all has only been partially characterised

(Raaijmakers and Weller, 1998). *Pseudomonas fluorescens* strain CHA0 has been also extensively studied and work has shown how the regulation of one of the metabolites (2, 4-diacetylphloroglucinol) produced by this strain occurs in suppression of black rot disease of tobacco and the take-all disease of wheat (Laville *et al.*, 1992).

A number of enhanced antibiotic producing derivative strains have been constructed from biological control strains, some of these have been shown to be able to better protect against the take-all disease in glasshouse experiments (Maurhofer *et al.*, 1992) and in the field (Peng and Ellingboe, 1990). However, none of these constructed strains has been commercialised.

Phenazine has been shown to be effective against take-all. The relative effectiveness of 2, 4-diacetylphloroglucinol producing strains in take-all biological control is poorly understood. Although both of these compounds are effective against take-all "*in-vitro*" and in glasshouse tests, in the field, the parent strain or the genetically modified strains are yet to be developed as a commercial inoculum.

Although other biological control agents have been tried, such as nonpathogenic strains of *Gaeumannomyces graminis* var. *graminis* (Wong *et al.*, 1996), they are not a feasible method for large scale control of take-all as there is no current technology available to grow such fungi on a large scale. The cost of producing such a fungal agent and to apply same in the field is prohibitively high compared to bacterial control agents. Therefore, bacterial control agents are the only feasible practical biological control method against take-all. The reason why any biological control against the take-all disease have not been utilised is that in very dry years its effectiveness can be decreased.

In work leading up to the present invention, the inventors identified a species of a non-fluorescent *Pseudomonas* and showed it to be effective against fungi and other pathogenic organisms.

The inventors have now identified the anti-pathogen component produced by the *Pseudomonas* species and have determined the biochemical and genetic pathway for its production. The

identification of this organism, the anti-pathogen agent and the cloning of the genetic sequences required for the component's production permits the development strategies for inhibiting plant pathogen infection and infestation.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

One aspect of the present invention provides an isolated strain of a non-fluorescent *Pseudomonas* species wherein said strain is capable of producing an anti-pathogen effective amount of a sugar acid wherein the type of said sugar acid is depended on the carbon source metabolized by said strain.

Another aspect of the present invention provides an isolated strain of *Pseudomonas* species AN5 capable of colonizing root rhizosphere of plants and wherein said strain of *Pseudomonas* species AN5 is capable of producing an anti-pathogen effective amount of a sugar acid wherein the type of sugar acid is dependent on the carbon source metabolized by said strain.

Yet another aspect of the present invention is directed to an isolated strain of *Pseudomonas* species AN5 capable of producing an anti-pathogen effective amount of D-gluconic acid from D-glucose.

Still another aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, an enzyme associated with the conversion of a carbon source to a corresponding sugar acid in a strain of non-fluorescent *Pseudomonas*.

Even still, another aspect of the present invention relates to an isolated nucleic acid molecule

comprising a sequence of nucleotides encoding:

(i) a glucose oxidase or an functional derivative thereof; and/or

(ii) a lactonase or functional derivative thereof;

wherein said nucleic acid molecule corresponds to non-contiguous genomic regions of *Pseudomonas* species AN5 and wherein both glucose oxidase and lactonase convert D-glucose to D-gluconic acid.

Still yet, another aspect of the present invention provides a composition comprising a sugar acid and optionally one or more carriers, excipients and/or diluents for use in controlling pathogen growth or infestation in or on plants or plant material.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Is a photographic representation showing biological activity of the anti-take-all compound from crude extract of *Pseudomonas* strain AN5 on TLC plate with (a) 10% w/v methanol in Chloroform (b) 30% w/v methanol in chloroform (c) 50% w/v methanol in Chloroform against take-all. This is an agar overlay assay on the TLC plate. The take-all fungus is plated on the top of the TLC plate in potato dextrose overlay agar and has the ability to grow well. The arrow indicates the single inhibition zone seen on these TLC plates where take-all is unable to grow because of an anti-fungal agent produced by a strain AN5. Note this anti-fungal compound remains at the origin and does not move with any of these solvents.

Figure 2a Is a photographic representation showing separation of compounds in crude extracts of *Pseudomonas* strain AN5 (a) and mutant strain AN5-MN1 (b) with *n*- propanol-ethyl acetate-water (5:2:3) on TLC plate of silica gel 60 F_{254}. The compounds separated by this solvent system are generally simple sugars. A change in the simple sugars produced by the mutant strain is observed compared to the parent strain.

Figure 2b Is a photographic representation showing agar overlay bioassay on TLC plates (Figure 2a) with take-all fungus. There is only one spot of clear inhibition against take-all (indicated by arrow) in *Pseudomonas* strain AN5 (a). The mutant strain (b) has lost this zone

of inhibition.

Figure 3a Is a photographic representation showing a thin layer chromatography of column fractions of AN5 on silica run with n-propanol-ethyl acetate-water (5:2:3). The numbers indicate the fraction number collected from the silica column. C indicates the complete crude extract used for the silica column. Compounds are observed eluting from fraction 9 collected from the silica column.

Figure 3b Is a photographic representation of inhibition of take-all fungus with fractions from silica column run with n-propanol-ethyl acetate-water (5:2:3) on PDA plate shown in Figure 3a. Fractions 17 to 20 are active and fractions 19 and 20 show the highest activity.

Figure 4 Is a photographic representation showing the column purified fraction of Figure 3 further purified by running out on TLC using the same solvent conditions. Bands observed were eluted in four samples (based on Rf value) and tested for biological activity on PDA plate against take-all using an agar overlay assay. Only the bands eluted in sample 3 (Rf value 0.75) are active.

Figure 5 Is a graphical representation showing ^{1}H NMR spectra of an active fraction purified by silica column and TLC as indicated in Figures 3 and 4.

Figure 5b Is a graphical representation showing ^{13}C NMR spectra of active fraction purified by silica column and TLC as indicated in Figures 3 and 4.

Figure 5c Is a photographic representation showing mass spectra of active fraction purified by silica column and TLC as indicated in Figures 3 and 4.

Figure 6a Is a photographic representation showing biological activity of pure gluconic acid (Sigma Pty. Ltd.) against take-all using an agar overlay assay. The concentrations of gluconic acid used are: (1) 25mg; (2) 15mg; (3) 12.5mg; (4) 7.5mg. The greater the concentration of gluconic acid, the greater the inhibition zone against take-all.

Figure 6b Is a photographic representation showing a biological activity of sugar acids (Sigma Pty. Ltd.) against take-all using an agar overlay assay. The concentration of sugar acids used is 12.5mg. The sugar acids used are: (1) malic acid; (2) ascorbic acid; (3) glutaric acid; (4) glucuronic acid. All four acids produce strong inhibition zones against take-all.

Figure 7 Is a photographic representation showing identification of 2,4-diacetylphloroglucinol by production of red colour pigment on king's B media. (a) *Pseudomonas fluorescens* Pf-5 (b) Pseudomonas strain AN5. Note that strain Pf 5 produces strong red pigment but strain AN5 does not. This shows that AN5 does not produce any 2,4-diacetylphloroglucinol.

Figure 8 Is a graphical representation of ^{1}H NMR of phenazine-1-carboxylic acid, an antifungal agent against take-all.

Figure 9 Is a graphical representation of ^{1}H NMR of crude extracts of Pf5 (a) AN5 (b) from malt agar.

Figure 10a Is a photographic representation of Potato Dextrose Agar with the indicator dye Bromocresol Purple added (plate a). Plate b. shows this medium with the parent strain AN5 streaked on it. The medium has changed colour to yellow and this is an indication of acidification of media. However, this does not happen when the mutant strain AN5-MN1 is streaked on the same medium (plate c). The medium goes more alkaline as indicated by the change in the colour of the media to purple.

Figure 10b Is a photographic representation showing take-all grown on Potato Dextrose Agar with the indicator dye Bromocresol Purple added. There is a purple hallow around the take-all as it grows suggesting it is releasing compounds which increase pH of the media.

Figure 11 shows the nucleotide DNA sequence of PQQ D gene of *Pseudomonas* strain AN5 (pggan5).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS.

The present invention is predicted in part on the elucidation of the anti-pathogen properties of a non-fluorescent species of *Pseudomonas*. The preferred pathogens in accordance with the present invention include eukaryotic and prokaryotic organisms. Most preferably, the pathogen is a fungus.

Accordingly, one aspect of the present invention provides an isolated strain of a non-fluorescent *Pseudomonas* species wherein said strain is capable of producing an anti-pathogen effective amount of a sugar acid wherein the type of said sugar acid is depended on the carbon source metabolized by said strain.

In accordance with this aspect of the present invention, when the carbon source is D-glucose, the sugar acid is predominantly D-gluconic acid. However, when the carbon source is, for example, galactose, the sugar acid is galactonic acid. Sugar acids contemplated by the present invention include but are not limited to gluconic acid, malic acid, ascorbic acid, glutaric acid, glutamic acid, glucuronic acid and galactonic acid.

The preferred isolate of *Pseudomonas* is exemplified by *Pseudomonas* AN5 (Nayudu *et. al*, 1994b). However, the present invention extends to a range of non-fluorescent *Pseudomonas* species or mutants or derivatives of strain AN5. Reference hereinafter to "*Pseudomonas* strain AN5" or its abbreviation "AN5" includes all other suitable non-fluorescent *Pseudomonas* species and their mutants and derivatives.

Accordingly, another aspect of the present invention provides an isolated strain of *Pseudomonas* species AN5 capable of colonizing root rhizosphere of plants and wherein said strain of *Pseudomonas* species AN5 is capable of producing an anti-pathogen effective amount of a sugar acid wherein the type of sugar acid is dependent on the carbon source metabolized by said strain.

More particularly, the present invention is directed to an isolated strain of *Pseudomonas* species

AN5 capable of producing an anti-pathogen effective amount of D-gluconic acid from D-glucose.

It is proposed herein that the sugar and in particular gluconic acid is effective against pathogens such as fungi of the genera Deuteromycete, Basidiomycete and Basidia. More particularly, it is effective against the following fungal species: *Epicoccum purpurescens, Alternaria species, Arthrobotrys oligosporus, Monilinia fructicola, Botrtis cinerea, Verticillium dahlia, Fomes annosus, Armillaria mellea, Boletus granulatus, Polyporus sulphureus, Saccharomyces cerevisiae*. It is also effective against a range of grass, positive and negative bacteria.

Reference herein to "mutants" and "derivatives" of *Pseudomonas* organisms include mutants with altered root rhizosphere colonizing ability (increased or decreased), altered metabolic ability (eg. increased or decreased ability to produce a sugar acid), auxotrophic mutants, replication mutants and recombinant mutants (eg. carrying additional genetic material or carrying extra chromosomal plasmids or DNA).

Particularly useful mutants include mutants having altered exopolysaccharide (EPS) production capacity. EPS production by bacteria influences how efficiently anti-pathogen agents like gluconic acid are transported to the site of a pathogen. Strains producing less EPS tend to be more efficient in inhibiting pathogen growth.

Other particularly useful mutants include transposon mutants. Transposon mutants are also useful as markers for the cloning of genetic material capable of encoding molecules required to catalyse or otherwise facilitate the conversion of a carbon source to a corresponding sugar acid.

Accordingly, another aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, an enzyme associated with the conversion of a carbon source to a corresponding sugar acid in a strain of non-fluorescent *Pseudomonas*.

Preferably, the *Pseudomonas* is *Pseudomonas* AN5.

Preferably, the sugar acid is D-gluconic acid from D-glucose although the present invention extends to other sugar acids, such as but not limited to malic acid, ascorbic acid, glutaric acid, glutamic acid, glucuronic acid and galactonic acid.

This aspect of the present invention is further described with reference to the production of D-gluconic acid.

Pseudomonas AN5 comprises two non-adjacent genomic regions which comprise the structural genes which code for the enzymes which catalyse the reactions for the production of D-gluconic acid. These structural genes include glucose oxidase and lactonase. Transfer of this region to other microorganisms such as other *Pseudomonas* strains, which don't produce gluconic acid or any other anti-fungal metabolite, leads to these strains now being able to produce an anti-pathogen metabolite and inhibit pathogens such as fungi. Two steps are required for converting D-glucose to D-gluconic acid. The genetic data are consistent with a small number of genes required for this process. The genes involved in the production of D-gluconic acid in *Pseudomonas* strain AN5 useful, therefor, for producing anti-pathogen activity in plants and other microorganisms.

Accordingly, another aspect of the present invention relates to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding:

(i) a glucose oxidase or an functional derivative thereof; and/or

(ii) a lactonase or functional derivative thereof;

wherein said nucleic acid molecule corresponds to non-contiguous genomic regions of *Pseudomonas* species AN5 and wherein both glucose oxidase and lactonase convert D-glucose to D-gluconic acid.

As the nucleotide sequence encoding glucose oxidase and lactonase are from non-contiguous regions of the genome, the present invention extends to fusion nucleotide molecules comprising a single nucleotide sequence encoding separately a glucose oxidation and a lactonase or their functional derivatives. Such a fusion nucleotide molecule is useful for introducing the trait of D-gluconic acid production from D-glucose into microorganism and plants.

The present invention further provides nucleic acid molecules encoding regulatory regions or genes which modulate, control or facilitate D-glucose to D-gluconic acid conversion. Such regulatory regions or genes include promotors, 3′ and 5′ regulatory regions, regulatory genes, operator regions or confronter genes. For example, the present invention provides a nucleic acid molecule encoding all of part of the operon required for production of the genome co-factor, pyrroloquinoline quinone (PQQ). The nucleotide sequence encoding the latter molecules is set forth in Figure 11. The present invention extends to this nucleotide sequence as well as sequence having at least 50% similarity thereto or a sequence capable of hybridizing thereto under low stringency conditions at 42°C. The latter nucleotide sequences are also capable of encoding PQQ or homologue thereof.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity. Any number of programs are available to compare nucleotide and amino acid sequences. Preferred programs have regard to an appropriate alignment. One such program is Gap which considers all possible alignment and gap positions and creates an alignment with the largest number of matched bases and the fewest gaps. Gap uses the alignment method of Needleman and Wunsch (1970) (*J. Mol. Biol. 48:* 443-453). Gap reads a scoring matrix that contains values for every possible GCG symbol match. GAP is available on ANGIS (Australian National Genomic Information Service) at website http://mel1.angis.org.au.

Reference herein to a low stringency at 42°C includes and encompasses from at least about 1% v/v to at least about 15% v/v formamide and from at least about 1M to at least about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which

includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5M to at least about 0.9M salt for hybridisation, and at least about 0.5M to at least about 0.9M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about 0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41$ (G+C)% (Marmur and Doty 1962 *J. Mol. Biol. 5:* 109). However, the T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (Bonner and Laskey (1974) *Eur. J. Biochem. 46:* 83, 1974).

Although not intending to limit the present invention to any one theory or mode of action, it is proposed that *Pseudomonas* AN5 or other organism or plant carrying the nucleotide sequences hereinbefore described oxidizes an aldose such as D-glucose at the aldehydic carbon atom by specific enzymes to form the corresponding carboxylic acid, which is generically called an aldonic acid. In the case of D-glucose, D-gluconic acid is yielded through oxidation of C-1 of aldose form. The first step catalysed by glucose oxidase (GOD) is an oxidation step and involves conversion of D-glucose to glucono-delta-lactone (GDL). The second step involves hydrolysis of GDL to gluconic acid by a lactonase. This last step is proposed to occur partially through non-enzymatic hydrolysis of the lactone. In this case, PQQ is likely to be an essential cofactor for one or both of these enzymes.

The present invention further contemplates genetic constructs comprising single of multiple vectors which singularly or in combination permit D-gluconic acid production (or other sugar acid) from D-glucose. Such genetic constructs are useful for introducing this trait to other organisms such as plants.

Accordingly, another aspect of the present invention provides a transgenic plant comprising single or multiple nucleotide sequences which encode enzymes and optionally regulatory regions required to convert a carbon source into a sugar acid.

Preferably, the carbon source is D-glucose and the sugar acid is D-gluconic acid.

Preferably, the nucleotide sequences are obtainable *Pseudomonas* species AN5 or a mutant or derivative thereof.

Such a transgenic plant is generally now fungal resistant or at least retards fungal infestation.

Plants contemplated by this aspect of the present invention include any plant sensitive to fungal infection such as chocolate spot, take-all and other plant pathogenic fungi. Preferred plants include beans, clover, rice, wheat, sugarbeet, cucumber and cotton and related plants. Most preferably, the plants are beans, clover, rice or wheat.

The nucleotide sequence may also comprise genetic elements useful for tissue specific or developmental specific expression of the enzymes and other molecules required for sugar acid production. Preferably, expression occurs at least in root tissue.

The present invention further extends to seeds and reproductive material for the transgenic plants herein described.

The present invention further contemplates the use of a sugar acid in the manufacture of a pathogen retardant for plants.

Preferably, the sugar acid is D-gluconic acid.

In accordance with this aspect of the present invention, D-gluconic acid or other sugar acid, is useful in a composition of matter to inhibit pathogens such as fungus growth or infestation.

Accordingly, another aspect of the present invention provides a composition comprising a sugar acid and optionally one or more carriers, excipients and/or diluents.

Preferably, the carrier, excipient or diluent is present in the composition.

The composition may be in a form suitable for spraying onto a plant or administered by way of

a power or other solid form. It may also be administered during ploughing or as part of the fertilizer procedure in crop care.

The term "pathogen" is used in its broadest sense to include any microorganism, such as a bacterium, fungus or yeast which infects a plant. The pathogen may, however, not cause a debilitating disease condition.

EXAMPLE 1

METHODS

Microbiological and Molecular biological methods

Standard methods were employed as described by Nayudu and Holloway, (1981), Nayudu and Rolfe (1987), Nayudu *et. al.*, (1994a) and Nayudu *et. al.*, (1994b)

Extraction of anti-fungal compound

Bacteria were grown at 25°C in 250ml flasks for two days in potato dextrose broth. The 150 ml isopropanol was added to 90ml of inoculated broth. The solution mixture was shaken for 15 minutes and then centrifuged for 10 minutes at 5000 revolutions per minute. Supernatant fluid was taken out and evaporated with rotavapor at 40°C. Approximately 100ml of ethanol (30ml three times) was used in an attempt to dissolve the crude. To the ethanol insoluble triturate, 50ml water was added, followed by 50ml acetone, leading to the precipitation of proteins.

Standardisation of thin layer chromatography (solvent system)

The resulting suspension was centrifuged and a total of 0.02ml., drawn from the supernatant as well as from ethanolic solution, was applied by a micropipette (Gilson Pipette) to the silica gel G F254 TLC plate (Aszalos *et al.*, 1968). According to this method, crude extract was first analysed in three solvent systems:1. methanol; 2. 10% w/v methanol in chloroform; 3. chloroform. Subsequently various other solvent systems such as pyridine-water (1:1), pyridine-water-absolute ethanol (1:1:1), pyridine-water-absolute ethanol (1:1:3), 2-propanol-water (17:3, 4:1, 7:3); acetone-water (9:1); acetone-*n*-butanol-acetic acid-water (8:0.5:0.5:1); *n*-propanol-water (7:1,7:1.5);*n*-propanol-ethyl acetate-water (5:1:4, 5:2:3, 5.5:2:2.5); methyl acetate-2-propanol-water (18:1:1); 2-propanol-ethyl acetate-water (1:1:2 and 6:1:3) were also tried to improve the resolution on silica TLC plates.

Purification of antifungal compound

1. Thin layer chromatography - The crude extracts were run on TLC plates with the standardised solvent system and different bands were scratched and extracted the compounds to analyse their activity on PDA (Potato Dextrose Agar) plates. The bioassay was also done on

TLC plates to confirm the band of interest.

2. Column chromatography - After selecting the best solvent system from TLC plate the silica column was run as described by Still *et al.* (1978). The biologically active crude extract of strain AN5, and of mutants AN5-MN1 and AN5-MN2 were separately run on silica columns. All the column fractions were run on TLC plates and assayed for biological activity on PDA plates. The active fractions, which have similar active band on TLC plates, were bulked for further analysis.

Analysis of biologically active fraction

The two biologically active bacterial strains Pf5 and AN5 were analysed for presence of 2, 4-diacetylphloroglucinol according to Keel *et al.*, (1996). The extracts of bacterial strains were analysed for the presence of amine, sugars and carbonyl with ninhydrin, Silver nitrate and dinitrophenylhydrazine test respectively. The active fractions from AN5 were further analysed by 1H NMR, 13C NMR, mass spectra to speculate the structure of the compound.

Bioassays

The agar plug assay (Poplawsky *et al.*, 1988) was used to test biocontrol of bacterial strains against various fungi. In the case of the take-all fungus, the inventors have designed an agar overlay assay to test activity of specific fractions. This was done by macerating the take-all fungus grown in Potato Dextrose broth and seeding it to Potato Dextrose Agar overlay. This was then poured onto thick Potato Dextrose Agar plates. The fractions were spotted on top, dried and incubated at 16°C for 3-5 days. To test biological control on TLC plates the Potato Dextrose Agar overlay (seeded with take-all, as described above) was poured onto the TLC plate and incubated for a week at 16°C.

EXAMPLE 2

FIELD TRIALS

In small scale field trials, the inventors were able to consistently increase wheat yield at different dry-land trial sites from between 12% to 36% due to the biological control of the take-all fungus by *Pseudomonas* strain AN5 (refer to Table 1).

Table 1

Summary of wheat yields (Kg/plot)

Trial △	AN5	Ba_1	Ba_2	Ba_3	Pf5	Control
Goolgowi	10.23	11.00*	n.t	n.t	8.86^	9.07
Forbes	n 10.76	10.28	11.28*	10.95*	9.70^	9.12
West Wyalong	5.70*	5.88*	5.17	5.41	4.65^	4.32

Legend

n.t not tested

^ not significant

* significant at the 99.9% confidence level

not marked - significant at the 95 % confidence level

△ geographical area in New South Wales, Australia

The Harden District Rural Services group (HRS) set up large scale trials with *Pseudomonas* strain AN5 in acre plots at a trial site at Harden. There was significant take-all disease at this site. The inventors found that the treated plot monitored through the wheat growing season had 30 to 40% suppression of disease. This was determined by the level of white head formation and visual scoring of symptoms on the roots. The wheat in these trials was harvested using a harvester which measured the amount of grain being harvested per second linked to a global positioning system (GPS). The GPS accurately determined the location of the segment of the plot being harvested from satellite positioning. This led to a colour wheat yield map of the trial site which was then analysed using NIH image analyser. The inventors found that there was 20% increase in wheat yield due to biological control protection by *Pseudomonas* strain AN5.

Survival of bacteria on the roots and in the soil is one of the most crucial factors in effective biological control protection. A typical survival pattern of the bacteria on the roots of wheat over a season is that they decrease in numbers. The rate of decrease is related to the moisture content of the soil. Similar results were observed in different soil types at different field sites over a number of seasons. High numbers of the bacteria build up at the start of the season (approximately 10^6 - 10^7 per gram of wheat root), with the numbers dropping off towards the

drier end of the season. In very dry years, a much more dramatic fall in the numbers of bacteria is observed on the wheat root and this correlates well with loss of biological control protection.

EXAMPLE 3

GENETICALLY ENGINEERED STRAINS

The inventors have genetically engineered new strains of *Pseudomonas* AN5 bacteria which produce more anti-fungal compound. This has been done by, for example, introducing a multi-copy plasmid with extra anti-fungal genes, transposon insertions or constructing strains which produce less EPS so more anti-fungal agent is released. These constructs show a greater inhibition zone in agar plate bioassays. These strains have been tested for biological control protection against take-all in controlled environment cabinet trials.

On particularly useful method is referred to herein as "M1". This is a single transposon mutant strain of AN5 which has lost anti-fungal activity. There is a transposon insertion in one of the anti-fungal genes. Into this strain, a pLAF 3 cosmid containing a complementary wild-type region of the mutant site was transferred. This plasmid is generally multi-copy in *Pseudomonas*, although copy number is relatively low (10-15 per chromosome). Strain M1 only poorly colonises the roots of wheat and there are very few numbers of bacteria present on the roots compared to when the parent strain (ie. AN5) is used. However, strain M1 protects against take-all fungus just as well as the parent strain. This means that significantly fewer number of bacteria of this genetically engineered strain on the roots are providing better protection compared to the parent strain. This shows that by increasing the anti-fungal compound production, a more effective biological control strain is created.

The inventors have created a range of genetically engineered strains which colonize the roots of wheat in a normal manner and show a greater inhibition zone in agar plate bioassays. The inventors have genetically engineered a number of biological control strains (Table 2) to produce higher amounts of a anti-fungal agent either by introducing multi-copy plasmids (eg. strain M1 and P1) or by obtaining a transposon mutant of this strain (eg. strain T5). These enhanced biological control strains were shown to be more potent against take-all in agar plate bioassays

by their enhanced clearing zones. Results with strains P1 and T5 indicated that an increase in biological control protection can be obtained against take-all fungus by increasing the anti-fungal nature of the strain without compromising colonisation ability. Therefore, an increased in anti-fungal activity increases biological control protection ability.

Testing these strains in glasshouse trials showed that they protect significantly better than the parent strain, AN5, as determined by the scoring of symptoms on the roots of wheat.

In such pot trials where there was an excess of take-all fungus artificially added, the inventors also showed that the enhanced more potent biological control strains gave significant protection against take-all for much longer periods of time than the parent strain AN5. The crucial aspect of these results is that these latter strains (ie. P1 and T5) which produce enhanced anti-fungal compounds have the potential to give greater protection when they are lower in number on wheat roots. Therefore, these strains should give better protection against take-all when they are reduced bacterial numbers on the roots of wheat in drier winter growing seasons.

TABLE 2

Pot trials for take-all protection: Comparison of genetically engineered strains.			
Bacterial Treatment	Bacteria (per gram of wheat root)	Take-all Disease (disease score)	Whole dry plant weight (% of normal control plant)
1. Control (no take-all)	0	0	1
2. Take-all treatment	0	5	9%
3. AN 5 (parent bacterial strain)	8×10^7	2	78%
4. AN 5:M1 (genetically engineered enhanced antibiosis with multi-copy plasmid)	1×10^3	2	83%
5. AN 5:P1 (genetically engineered enhanced antibiosis with multi-copy plasmid)	8×10^5	1	93%
6. AN 5:T5 (genetically engineered enhanced antibiosis transposon Tn *5* mutant)	5×10^7	1	95%

Legend

Treatment 1- Control, normal wheat with no take-all or bacteria added to any pots .

2 , 3, 4, 5 ,6- Take-all was added to all other treatments on millet seed.

2- take-all added but no biological control bacteria added to it.

Disease score for take-all is based on a scale we devised where 0 indicated no disease, 5 indicates the maximum disease (on crown of plant) In this case 1

indicates just detectable disease. At the end of the experiment the plant roots and shoots were dried and weighed. The results presented show the relative weights of treatments 2-6 in comparison to treatment 1 which was the control and considered to be the full potential growth of the plant. This is expressed as a percentage. The results presented are the average of six treatments. Enhanced antibiosis is enhanced anti-fungal production by the strain.

EXAMPLE 4

BIOLOGICAL CONTROL OF CHOCOLATE SPOT

Chocolate spot is the most significant disease of faba beans. Currently, fungicides are applied a number of times during the growing season.

Pseudomonas strain AN5 is effective against the Chocolate spot disease of faba bean (caused by the *Botrytis fabae* fungus) and is able to suppress the Chocolate spot fungus in bio-assays in the laboratory. Furthermore, sugar acids such as D-gluconic acid suppress the fungus in "*in-vitro*" bioassays.

Biological control protection against Chocolate spot in glasshouse experiments reduce the disease by up to 90%. In the glasshouse, the inventors were able to induce chocolate spot by spraying *Botrytis* fungus onto the plant. The symptoms were observable within a few days of spraying. If the plants were sprayed with biological control bacteria before or after the spraying of *Botrytis* fungus, a significant reduction in the chocolate spot disease was observed. A scale was devised for the Chocolate spot disease by visual scoring of the symptoms. Using this scale, it was shown that up to 95% disease could be obtained in the Chocolate spot disease (as indicated by the visual symptoms) with biological control spraying. For this disease, the inventors propose to create transgenic faba bean plants which produce sugar acids such as gluconic acid to induce resistance against chocolate spot disease.

EXAMPLE 5

THIN LAYER CHROMATOGRAPHY - SOLVENT SYSTEMS

The compound having anti-fungal activity in *Pseudomonas* strain AN5 against take-all fungus did not migrate from origin with methanol, 10% w/v methanol chloroform and chloroform, the three basic solvent systems using TLC. The biological activity was tested on TLC plates as well as after scratching bands and extracting the compounds from plates on PDA plates. There was no growth of take-all fungus on the origin of the TLC plate as well as on PDA plates with the same fraction (Fig. 1). The bioactive fungus compound having an Rf value of about 0.7 with pyridine-water-absolute ethanol (1:1:1) and Pyridine-water-absolute ethanol (1:1:3) was obtainable but the resolution was still poor. Silica gel Reverse Phase Thin layer chromatography with acetonitrile-methanol-water was unable to produce better resolution. As the above solvents gave poor resolution, the solvent systems usually applied for carbohydrates was also used. The elution systems in the chromatographic separation of sugars are organic solvents of binary or ternary composition. Water is an indispensable component, since water free solvents, or solvents having low water content, gives diffused spots (as with crude extracts of AN5 and mutants) which compromise the separation (Ghebregzabher *et al.*, 1976). From the solvent systems normally used to separate carbohydrates, only one system, *n*-propanol-ethyl acetate-water has been successful. The best separation of bands on TLC plate was achieved with *n*-propanol-ethyl acetate-water (5:2:3) (Fig. 2). This solvent mixture was normally used for separation of hexosesamines (Ghebregzabher *et al.*, 1976 and Gal, 1968). These results indicated that the anti-fungal compared was a carbohydrate-type molecule.

EXAMPLE 6

PURIFICATION OF THE COMPOUND

Adsorption chromatography on columns or on TLC plates is a useful method for separation and purification of simple sugars, sugar derivatives and oligosaccharides. However, this technique is time consuming and frequently results in poor resolution due to band tailing. Therefore, the inventors followed the substantially faster technique of "flash chromatography" to purify the

compound (Still *et al.*, 1978). Flash chromatography is basically an air pressure driven hybrid of medium pressure and short column chromatography. Having chosen the solvent system from the TLC plate results, the silica column was run as described by Still *et al.* (1978). Three different columns from crude extracts of strain AN5 and of mutant AN5-MN1 were run. All the column fractions were run on TLC (Fig. 3a) plates and were tested for activity on PDA plates (Fig. 3b). The fraction with an Rf of about 0.75 on TLC was active in the case of AN5 whereas the identical band in mutants were inactive which revealed the possibility of mixture of compounds having similar Rf values. Moreover, the column fractions could not be treated as purified compound as many bands were seen on TLC plates (Fig. 3a). The compound was further purified by running bulked active fractions of AN5 on TLC and bands were scratched from TLC and tested for activity against take-all on the PDA plate (Fig. 4).

EXAMPLE 7

ANALYSIS OF ACTIVE FRACTION

This biologically active fraction was analysed by 1H NMR, 13C NMR and mass spectra. 1H NMR of active fraction from TLC has peaks at 5.09, 3.57, 3.69, 3.39, 3.26, 3.69 and 3.63 which represents protons attached to carbon atoms 1, 2, 3, 4, 5, and 6, respectively and peaks for D-gluconic acid at 3.99, 3.89, 3.62, 3.62, 3.68 and 3.52 from C2 to C6 13C NMR of fraction has peaks at 94.67, 75.35, 74.07, 74.02, 72.23 and 63.16 for carbons 1 to 6 of alpha D-glucose, peaks 98.49,78.53, 78.34, 76.71, 72.19, and 63.32 for C1- C6 of beta D-glucose and peaks at 181.2, 76.67, 73.54, 75.16, 73.76 and 65.2 for C1 to C6 gluconic acid. Mass spectrum also had these peaks for D-glucose and D-gluconic acid. The analysis showed that this fraction had compounds D-glucose and D-gluconic acid (Fig. 5a, 5b and 5c). Therefore pure D-gluconic acid and D-glucose and some other sugar acids were tried for activity on PDA plates and it was found that sugar acids have strong biologically activity against take-all (Fig. 6).

EXAMPLE 8

IDENTIFICATION OF ACTIVE COMPOUND IN *PSEUDUMONAS* AN5

The nature of the compound produced by *Pseudomonas* AN5 is different than the compounds

produced by other bacterial strain. Some of the results indicating these differences are summarized below.

Based on solubility and coloured pigments: PCA is a pigmented greenish-yellow antibiotic which accumulated in culture of *P. fluorescens* strain 2-79 (Gurusiddaiah *et al*, 1986). The PCA is highly soluble in chloroform and methylene chloride and insoluble in water, methanol and ethyl acetate whereas the compound produced by AN5 is highly soluble in water and did not produce any coloured pigments while growing in media. Similarly, Phl producing strains produced red pigment on King's B medium (Keel *et al*, 1996) and differed from the compound produced by AN5 because no coloured substances were produced by it (Fig . 7).

Separation on TLC: Other compounds did not match the AN5 produced compound as this new compound could not be separated on TLC by using the solvents required to resolve previously identified compounds. The new compound appeared to have a carbohydrate nature since it could be resolved with solvents used to separate carbohydrate molecule. On thin layer chromatographic plate of silica gel with chloroform: Methanol (19:1), Phl runs with Rf value 0.2 and pyoluteorin with Rf value 0.5 (Keel *et al*, 1992). On reverse-phase C18 TLC with acetonitrile: methanol: water (1:1:1) Phl, pyoluteorin and pyronitrine moved with Rf values 0.88, 0.75 and 0.28 respectively (Rosales *et al*, 1995 and Pfender *et al*, 1993). The antifungal compound produced by AN5 did not move from origin with chloroform: Methanol (19:1) (Fig. 1) and resolved poorly with acetonitrile: Methanol: water (1:1:1).

Proton NMR spectra: In 1H NMR of PCA the peaks were only observed above 7 ppm as reported by Gurusiddaiah *et al.* (1986) [Fig. 8]. whereas no peaks were observed in this region in spectra of the new compound. In proton NMR of Phl, two peaks at about 6.00 and 2.5 ppm were obtained which represented attachment of one H to 6 carbon and six protons of acetyl group respectively (Keel *et al*, 1992) and this did not match with proton NMR of the compound produced by AN5 (Fig. 9).

The peaks observed in 13C spectra, 1H NMR and mass spectra of partially purified active fraction have provided evidence that the nature of new compound is of carbohydrate type in

particular a sugar acid (Fig. 5).

EXAMPLE 9

CONFIRMATION OF ACTIVE COMPOUND IN STRAIN AN5 BY CHARACTERISATION OF MUTANTS.

Three transposon mutants, AN5-MN1, AN5-MN2, and AN5-MN3 were isolated which have lost biocontrol activity, do not produce the anti-fungal metabolite and are unable to control the take-all root disease of wheat. The major difference in these mutants from the parent strain is that they cannot utilise glucose in a similar pathway. These mutants are deficient in an enzyme which converts glucose to sugar acid. The parent strain AN5 and mutants were streaked onto PDA supplemented with 0.015g of bromocresol purple per litre to verify the above mechanism. AN5 growth at 2% w/v glucose lead to medium acidification and produced a yellow halo whereas mutants were unable to produce a yellow halo after 3-4 days (Fig 7). The plates with the mutants on them went purple, which is an indicator of alkalinity. This colour change was the same as in all mutants. So the growth of mutants has increased the pH of PDA, whereas AN5 is decreasing the pH of PDA. This further supports the acidic nature of anti-fungal compound produced by AN5.

The effects of different concentrations of acids on the take-all root disease is shown in Table 3.

EXAMPLE 10

pH CHANGE INDUCED BY NOVEL COMPOUND

The take-all fungus was grown on potato dextrose plates with indicator dye. After 6-7 days a purple ring developed around take-all which is an indicator of alkalinity. This indicated that as the take-all fungal hyphae grow it releases compounds in to the media which are alkaline in nature so the growth of take-all has increased the pH of PDA, whereas AN5 decreases the pH of PDA which further supports the acidic nature of the antifungal compound produced by this organism.

EXAMPLE 11

PSEUDOMONAS STRAIN AN5 IS CAPABLE OF PRODUCING OTHER SUGAR ACIDS DEPENDING ON WHAT SUGAR CARBON SOURCE IS PROVIDED

Pseudomonas strain AN5 when grown on nutrient agar, Kings B medium or Malt agar does not produce any significant amount of sugar acids as indicated by the media becoming more basic. There was little or no biological activity in the extracts from these media when tested against the take-all fungus. There is very strong biological activity when *Pseudomonas* strain AN5 is extracted from potato dextrose medium which contains glucose as a carbon source. Furthermore, the pH of this medium turns acidic in nature by the production of gluconic acid. However, when strain AN5 is grown on potato agar (which contains starch as its main carbon source) there is no production of gluconic acid and the media turns more basic in nature. There is no anti-fungal biological control activity of extracts of *Pseudomonas* strain AN5 from potato agar. If a different carbon source is added to the potato agar such as galactose, then *Pseudomonas* strain AN5 produces an acid. There is biological activity against take-all fungus with strain AN5 extracted from the media. The acid produced in this case is galactonic acid and not gluconic acid. In a similar fashion mannose can be used as the sole carbon source leading to its corresponding acid. However, this biological activity is not as strong as when gluconic acid is produced. From what is known about the efficiency of conversion of different substrates by this process using glucose oxidase in *Aspergillus*, glucose is the most efficient substrate for conversion to its corresponding sugar acid. Although other sugars can be converted to their

corresponding acid using this enzyme system the procedure is not as efficient as in the case of the primary substrate glucose.

EXAMPLE 12

BROAD HOST RANGE OF BIOCONTROL BY ANTIFUNGAL AGENT PRODUCED BY STRAIN AN5

Pseudomonas strain AN5 was tested against a range of microorganisms for biological control as listed in Table 4. The anti-fungal agent produced by *Pseudomonas* strain AN5 showed activity against a broad spectrum of fungi which represent plant pathogens, human pathogens and saprophytes. When tested against bacterial species there were some Gram negative and Gram positive species which were inhibited by the agent produced by *Pseudomonas* strain AN5 but this was not as broad as in the case of fungi. There was a range of inhibition seen from total to partial.

EXAMPLE 13

RESTRICTION FRAGMENTS

Restriction enzyme fragments (e.g. *Bam*H1 , *Hind* III, *Eco*R 1) carrying the two genomic regions shown to be essential for the production of sugar acids were prepared from *Pseudomonas* strain AN5. One region produces the enzymes involved in catalysing the conversion of D-glucose to D-gluconic acid. The second region is responsible for synthesising the cofactor which is a cofactor required for the functioning of the enzymes which catalyse the reaction.

Region coding for enzymes which convert glucose to gluconic acid.

3.8Kb *Bam*H1

7.6Kb *Bgl* 11

6.5Kb *Pst*1

approx. 18Kb *Hind* 111

approx. 22Kb *EcoR*1

Region coding for cofactor PQQ essential for enzymes to be active.

8.0 Kb *Pst*1

approx. 18Kb *Hind* 111

approx. 20Kb *Eco*R1

approx. 20Kb *Bam*H1

Vectors employed

The following vectors are employed for cloning purposes:

pUC18

pUC 19

pUC 118

pUC 119

pLAF 3

pKT 240

DNA sequence

The DNA sequence of the essential genes required for catalysation of glucose of gluconic acid is determined. The presence of the gene encoding glucose oxidisation is determined by Southern hybridisation. The region coding for cofactor PQQ has been sequenced and is show in Figure 11.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Table 3 Effect of different concentration of acids on the take all root disease.

Acid	pH	Concentration (mg)	Inhibtion zone radius (cm)	Pka
Malic acid		7.5	1	3.4
		12.5	2	
	1.5	25	2.5	
Ascorbic acid		7.5	0	4.17
		12.5	0.9	
	1.9	25	2	
Glutaric acid		7.5	1.5	4.31
		12.5	2	
	2	25	2.5	
Glucuronic acid		7.5	0	–
		12.5	1	
	1.7	25	2	
Gluconic acid		7.5	0.7	3.6
(From sodium salt)		12.5	1.0	
	~5	25	2	
Gluconic acid		7.5	0	–
(sodium salt)		12.5	0	
	~7	25	0	
Galactouronic acid		7.5	0.7	
		12.5	2	
	1.7	25	2.5	
Acetic acid		1.05	1.5	4.76
		2.1	3.0	
		4.2	>3	
	2.4	6.2	>3	
		8.4	>3	

Pure gluconic acid from Sigma, in a liquid form, was found to be biologically active against take-all. However it was difficult to determine concentration as there was significant ester formation in this liquid, which was detected by mass spectroscopy. Therefore to obtain accurate concentration for biological activity the sodium salt of gluconic acid was used.

Pka and pH values suggest that malic acid is the strongest acid in these acids . But order of inhibition of take-all by these acids is not the same as the strength of the acids. Therefore it is not only the pH, but some other factors are also responsible for the inhibition of take-all.

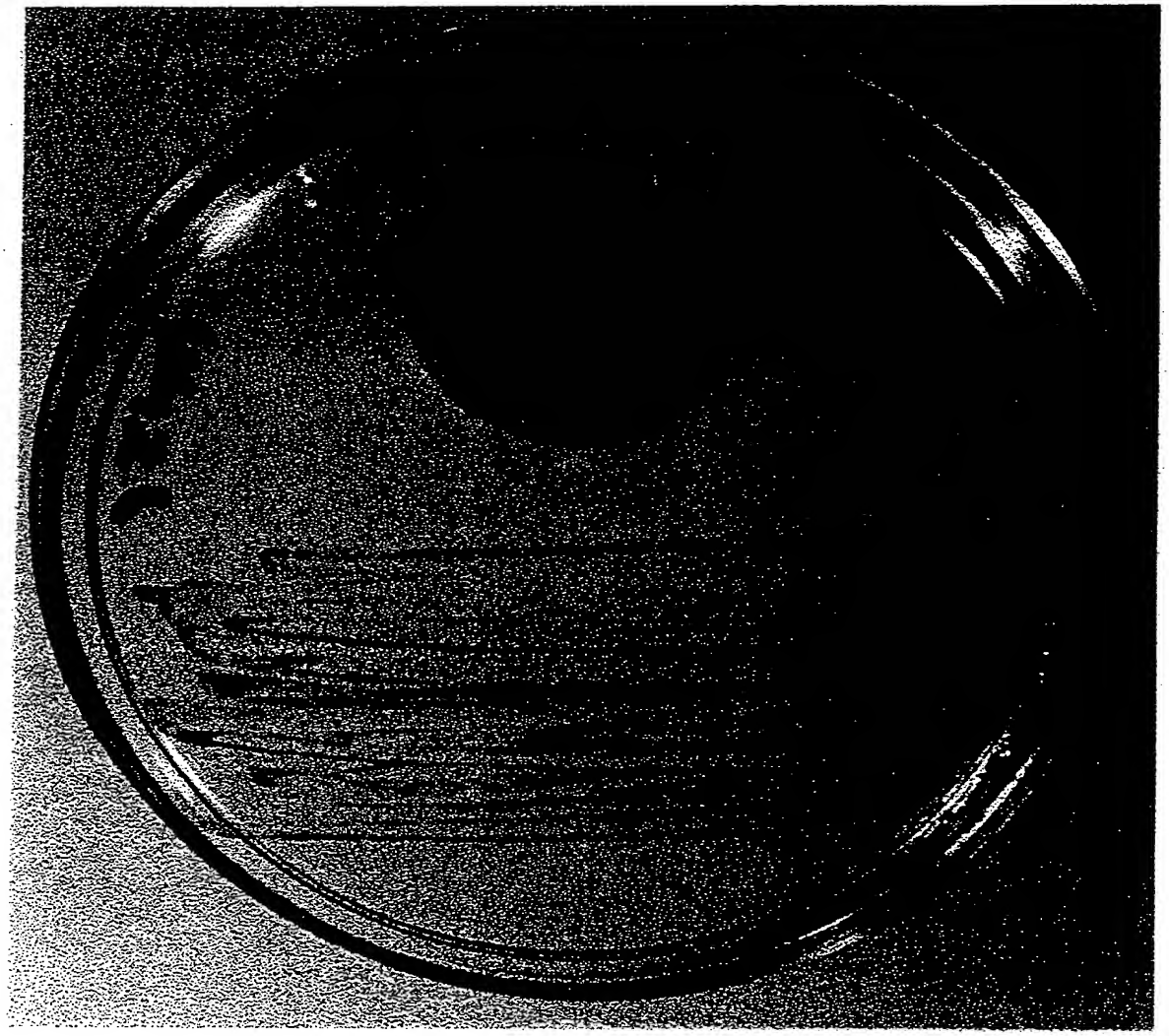
Table 4 Biological control strain AN5 protection against various microorganisms

Genera	Species tested	Type
Deuteromycete	*Epicoccum purpurescens*	saprophyte
Deuteromycete	*Alternaria species*	saprophyte
Deuteromycete	*Arthrobotrys oligosporus*	saprophyte
Deuteromycete	*Monilinia fructicola*	pathogen
Deuteromycete	*Botrtis cinerea*	pathogen
Deuteromycete	*Verticillium dahlia*	pathogen / saprophyte
Basidiomycete (bracket)	*Fomes annosus*	pathogen
Basidiomycete (toadstool)	*Armillaria mellea*	pathogen
Basidiomycete (toadstool)	*Boletus granulatus*	Mycorrhizal
Basidiomycete (toadstool)	*Polyporus sulphureus*	saprophyte
Yeast (Basidia)	*Saccharomyces cerevisiae*	saprophyte
Bacteria gram negative	number of species	many types
Bacteria gram positive	number of species	many types

The host range of anti-fungal agent gluconic acid produced by *Pseudomonas* strain AN5 was tested in a plate assay on Potato Dextrose agar. In the case of fungi tested strain AN5 completely inhibited the particular species. or slowed its growth rate significantly. A couple of examples of assays are listed in the attachment. In the case of bacteria only some species tested were significantly inhibited by the gluconic acid produced by strain AN5. Viruses and other micro organisms may also be inhibited by this agent, and will be tested.

Attachment Table 5

Agar plate assay showing significant slowing in growth rate of Fusarium species (causes ear rot and in corn)



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DATED this 29th day of January, 1999

THE AUSTRALIAN NATIONAL UNIVERSITY

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicants

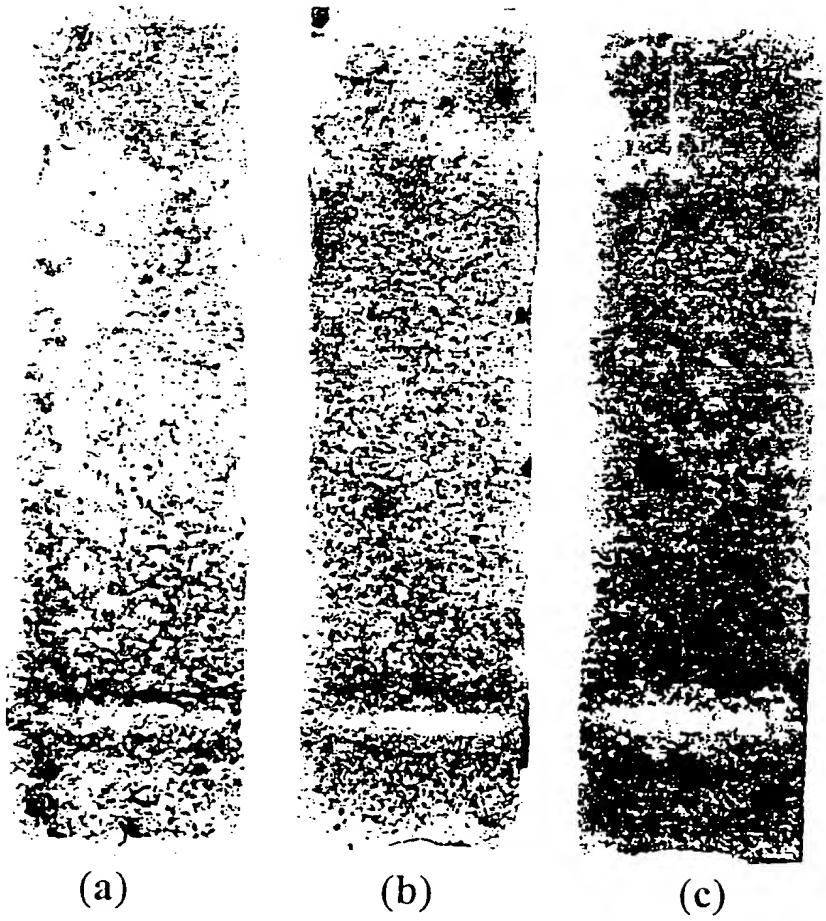
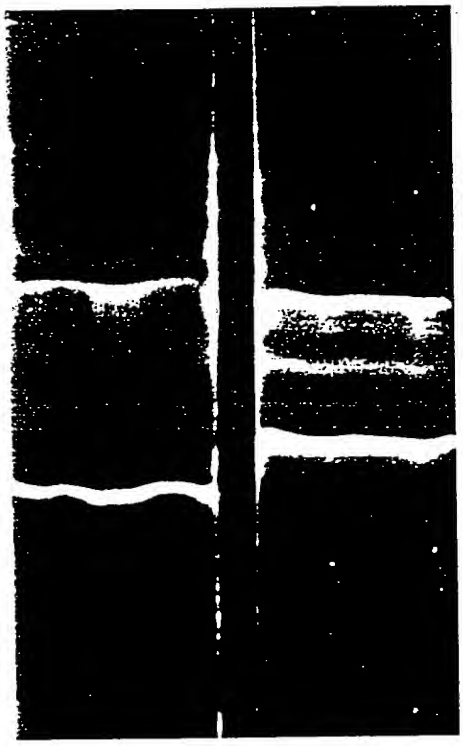
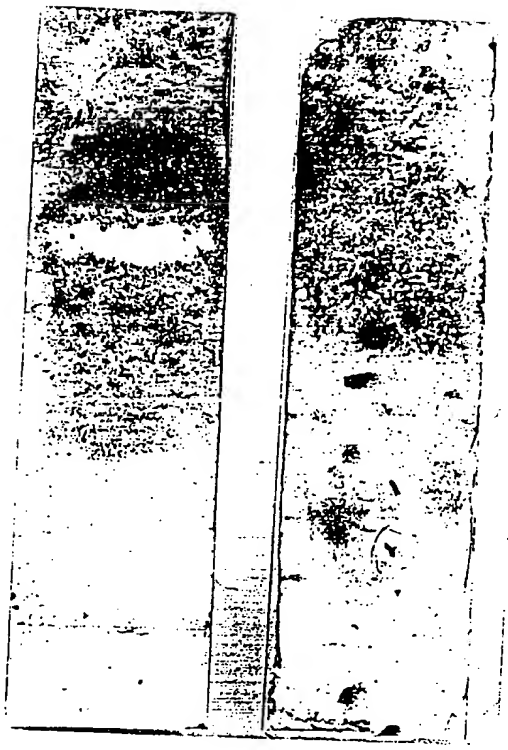


Figure. 1: Biological activity of the compound from crude extract of parent strain AN5 on TLC plate with (a) 10% methanol in chloroform (b) 30% methanol in chloroform (c) 50% methanol in Chloroform against take-all. This is an agar overlay assay on the TLC plate. The take-all fungus is plated on the top of the TLC plate in potato dextrose overlay agar and has the ability to grow well. The arrow indicates the single inhibition zone seen on these TLC plates where take-all is unable to grow because of an anti-fungal agent produced by strain AN5. Note this anti-fungal compound remains at the origin and does not move with any of these solvents.



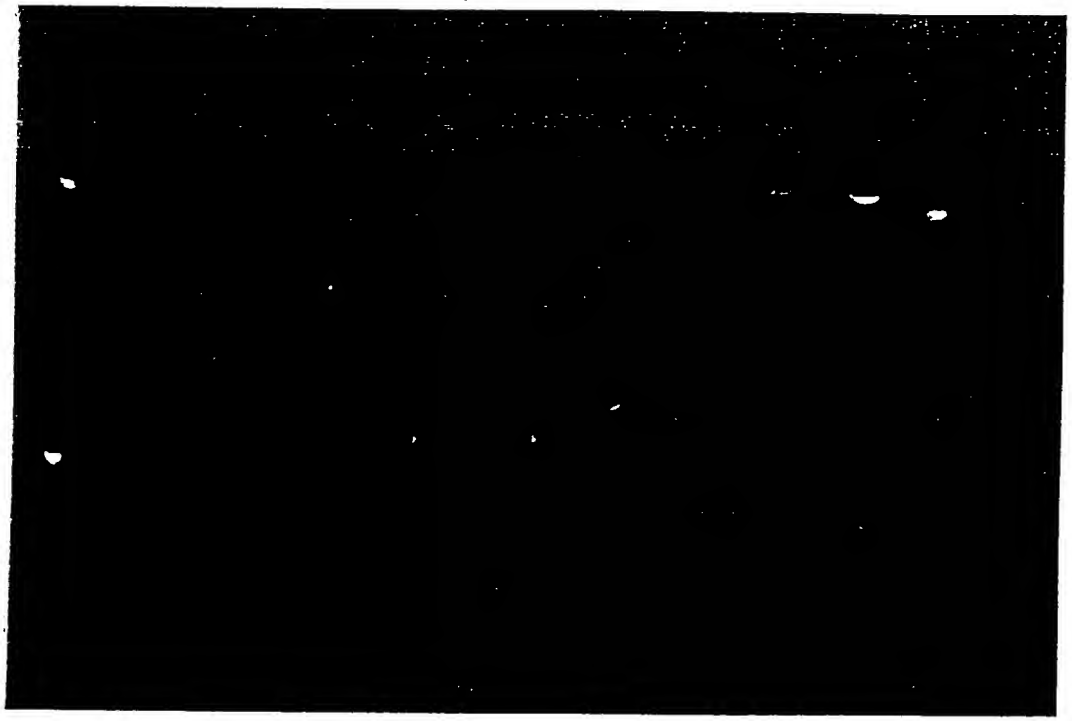
(a) (b)

Figure 2a: Separation of compounds in crude extracts of parent strain AN5 (a) and mutant strain AN5-MN1(b) with *n*- propanol-ethyl acetate- water (5:2:3) on TLC plate of silica gel 60 F_{254}. The compounds separated by this solvent system are generally simple sugars. You can see there has been a change in the simple sugars produced by the mutant strain compared to the parent strain.



(a) (b)

Figure 2b: Agar overlay bioassay done on TLC plates shown in Figure 2a with take-all fungus. There is only one spot of clear inhibition against take-all (indicated by arrow) in the parent strain (a). The mutant strain (b) has lost this zone of inhibition.



C 1 3 5 7 9 11 13 15 17 19 21 23

Figure3a: Thin layer chromatography of column fractions of AN5 on silica run with n-propanol-ethyl acetate- water (5:2:3). The numbers indicate the fraction number collected from the silica column. C indicates the complete crude extract that was used for the silica column. You can see compounds starting to be eluted from fraction 9 collected from the silica column.

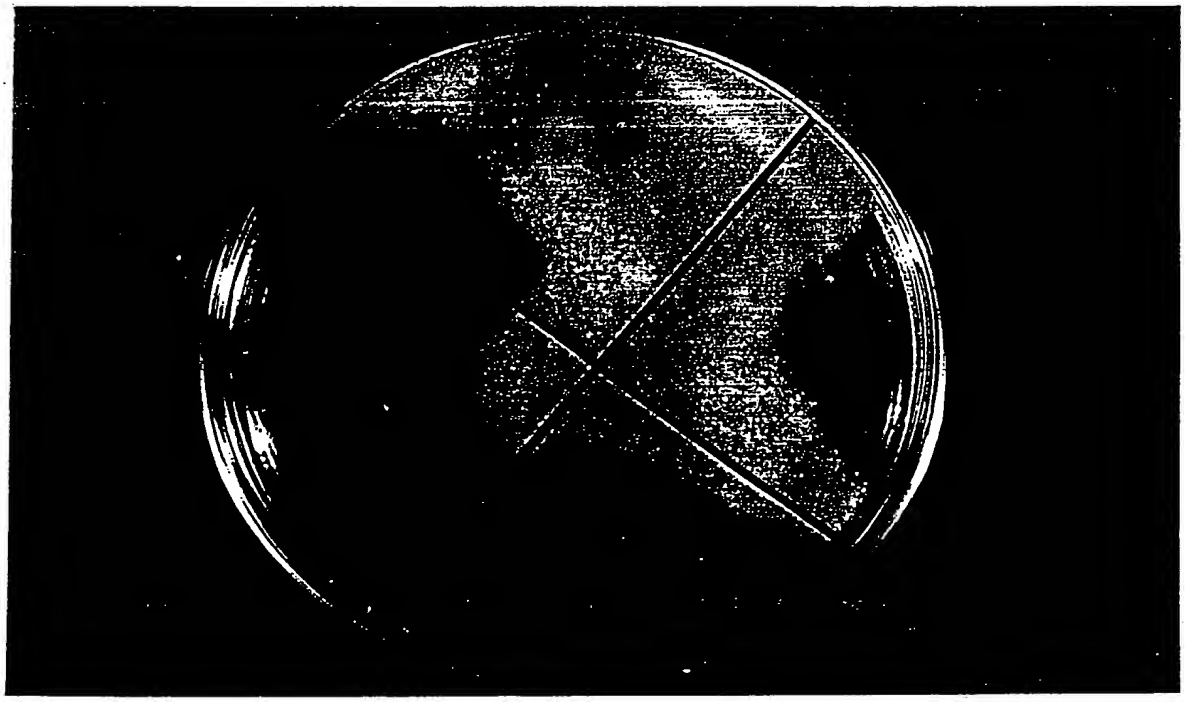


Figure 3b Inhibition of take-all fungus with fractions from silica column run with n-propanol-ethyl acetate- water (5:2:3) on PDA plate shown in Figure 3a. You can see fractions 17 to 20 are active, with fractions 19 and 20 showing the greatest activity.

Figure 4 The column purified fraction shown in Figure 3 were further purified by running out on TLC again using the same solvent conditions. Then bands observed were eluted in four samples (based on Rf value) and tested for biological activity on PDA plate against take-all using an agar overlay assay. Only the bands eluted in sample 3 (Rf value 0.75) are active.

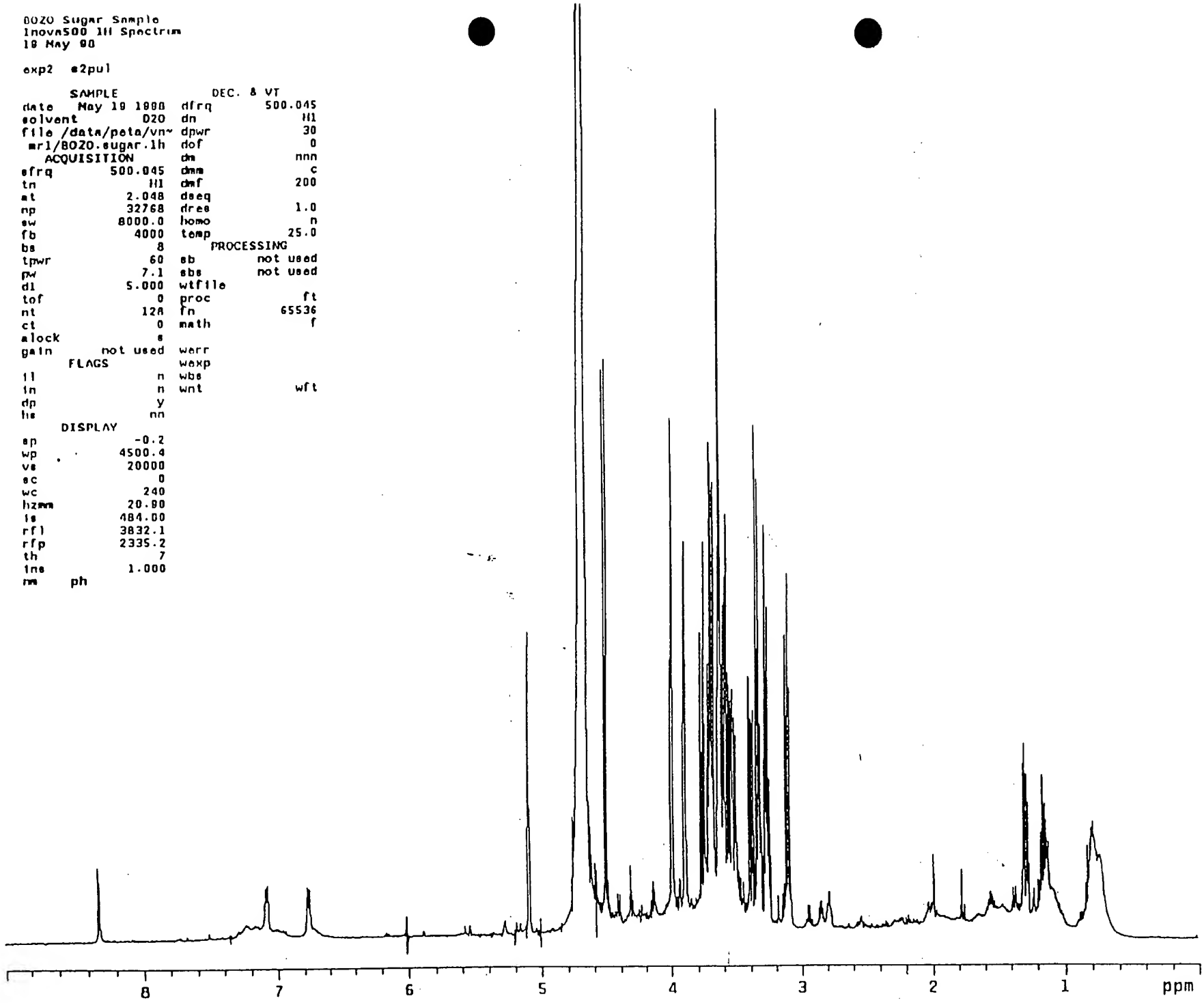


Figure 5 a ^{1}H NMR spectra of active fraction purified by silica column and then TLC sample as indicated in Figures 3 and 4.

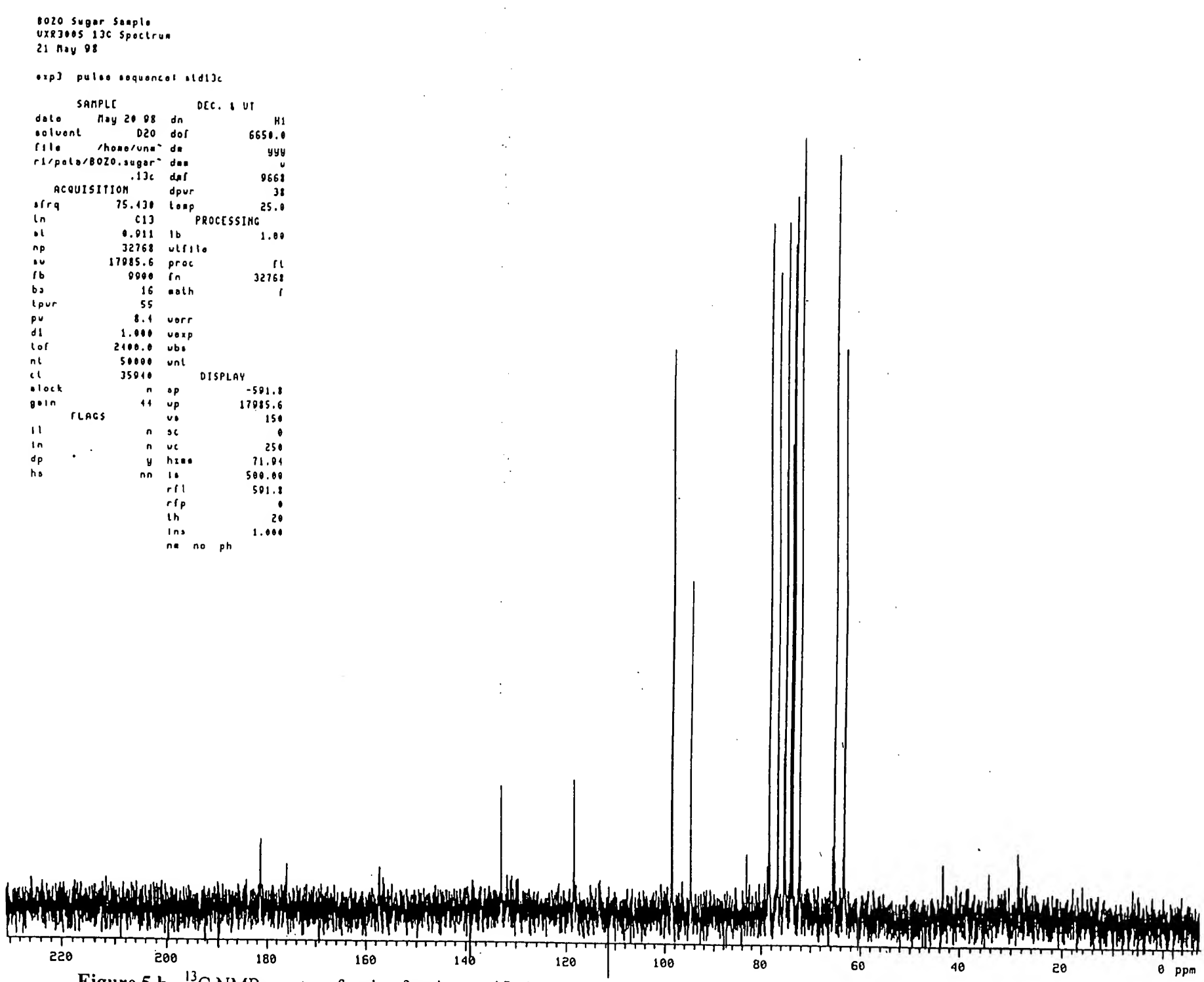


Figure 5 b ^{13}C NMR spectra of active fraction purified by silica column and then TLC sample as indicated in Figures 3 and 4.

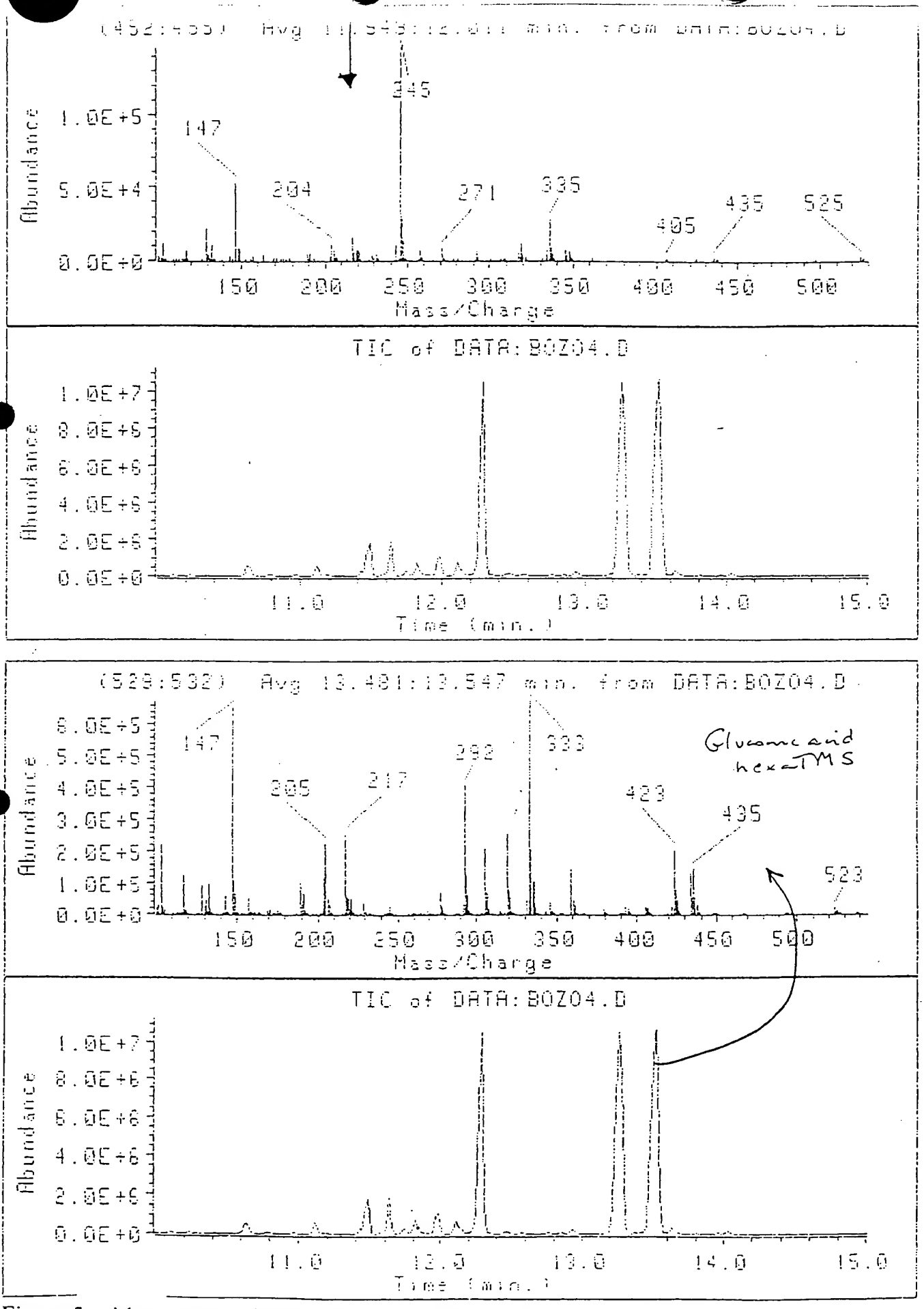


Figure 5 c Mass spectra of active fraction purified by silica column and then

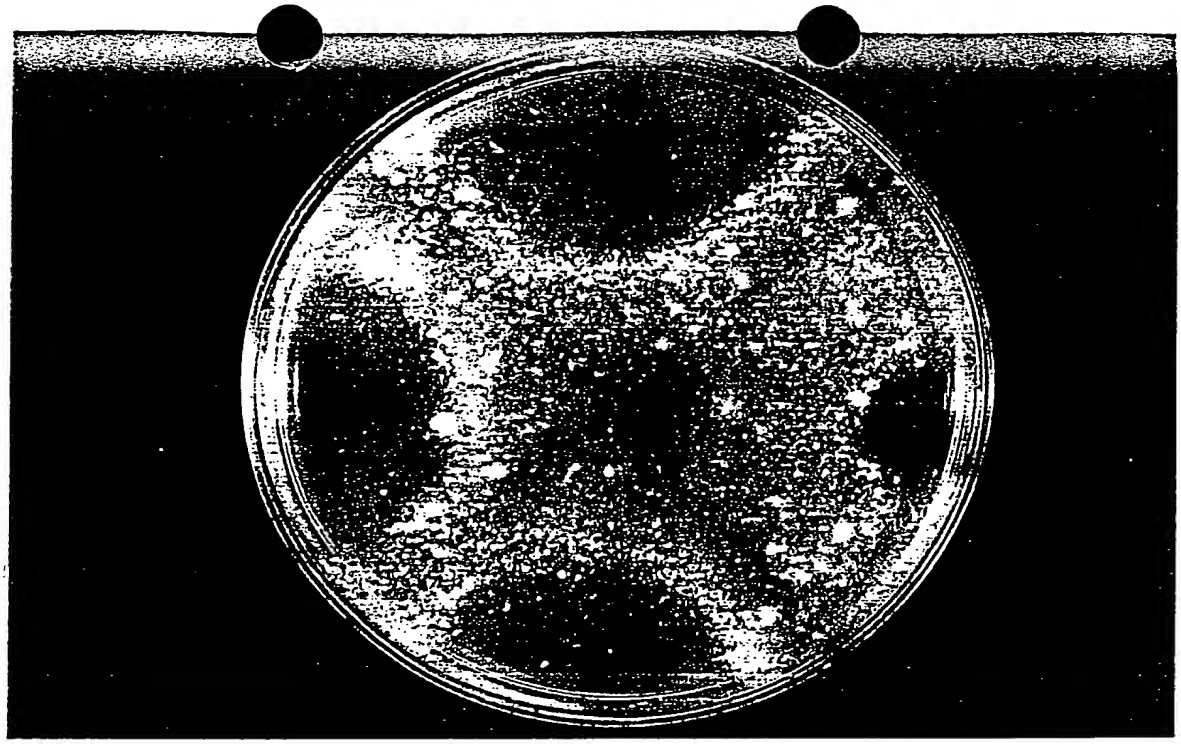


Figure 6a Biological activity of pure Gluconic acid from Sigma Pty Ltd. (found in *Pseudomonas* strain AN5) against take-all using an agar overlay assay. The concentrations of gluconic acid used are: (1) 25mg; (2) 15mg; (3) 12.5mg; (4) 7.5 mg. You can clearly see the greater the concentration of gluconic acid the greater the inhibition zone against take-all.

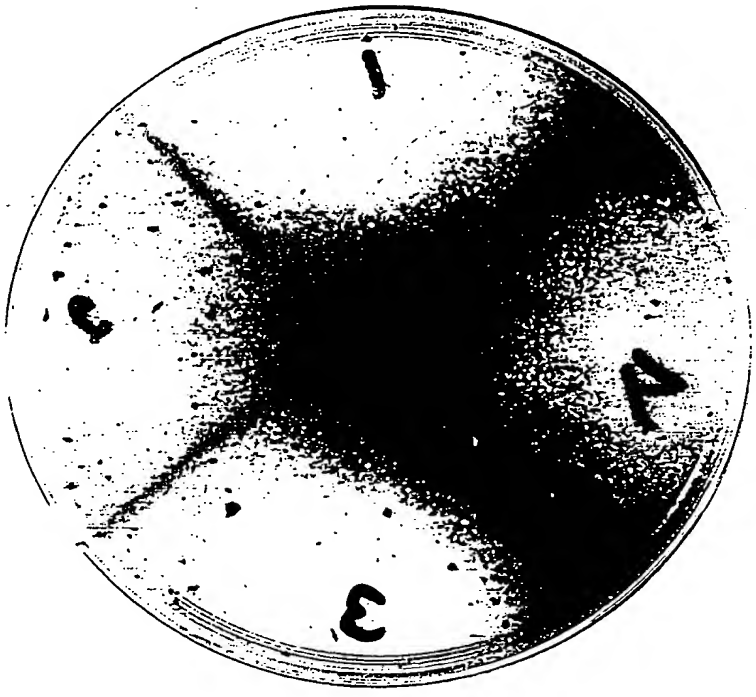


Figure 6b Biological activity of some sugar acids obtained from Sigma Pty Ltd. against take-all using an agar overlay assay. The concentrations of sugar acids used is 12.5mg. The sugar acids used are: (1) malic acid ; (2) ascorbic acid (3) glutaric acid; (4) glucuronic acid. You can clearly see all four acids produce strong inhibition zones against take-all.

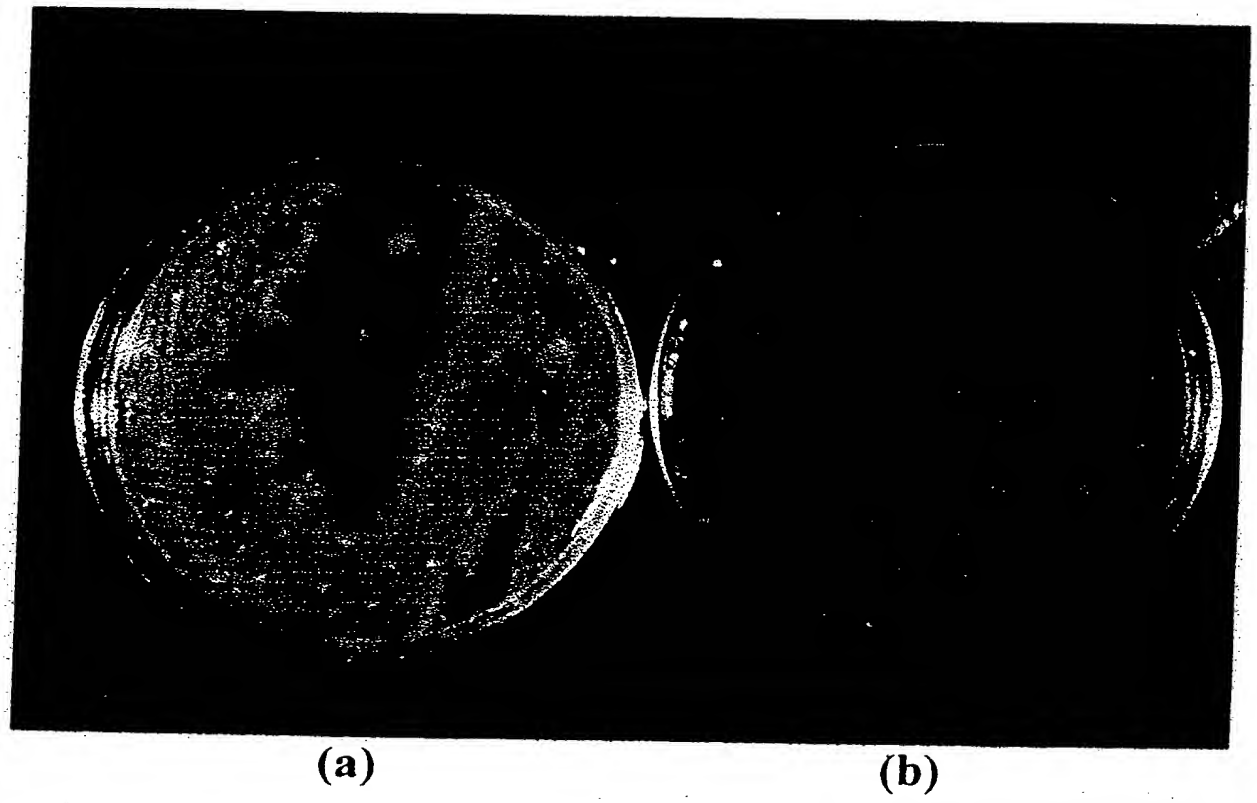


Figure 7 Identification of 2,4-Diacetylphloroglucinol by production of red colour pigment on king's B media. (a) *Pseudomonas fluorescens* Pf-5 (b) *Pseudomonas* strain AN5. Note that strain Pf 5 produces strong red pigment but strain AN5 does not. This shows that AN5 does not produce any 2.4-Diacetylphloroglucinol.

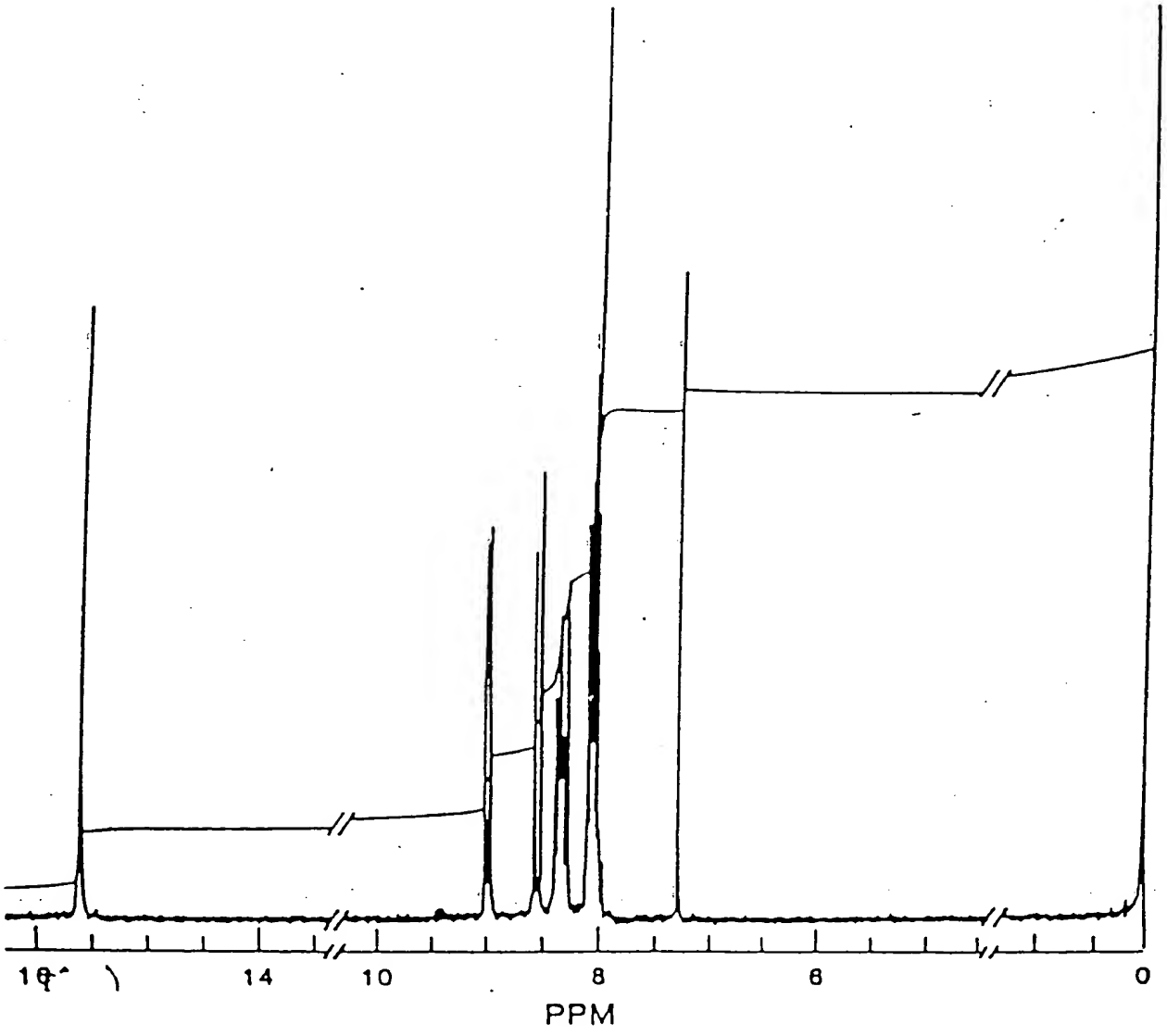


Figure 8: ^{1}H NMR of phenazine-1-carboxylic acid, an antifungal agent against take-all

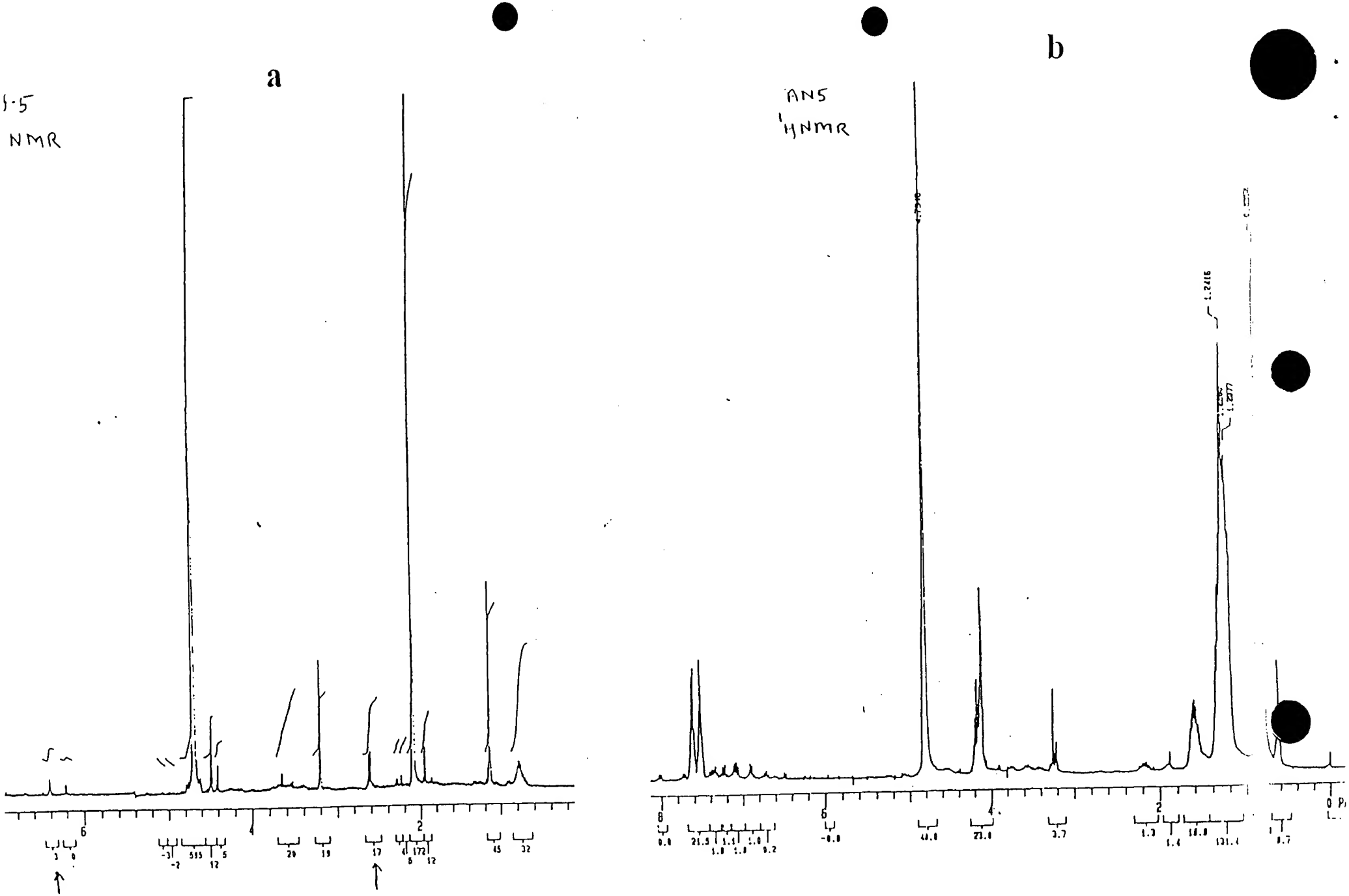
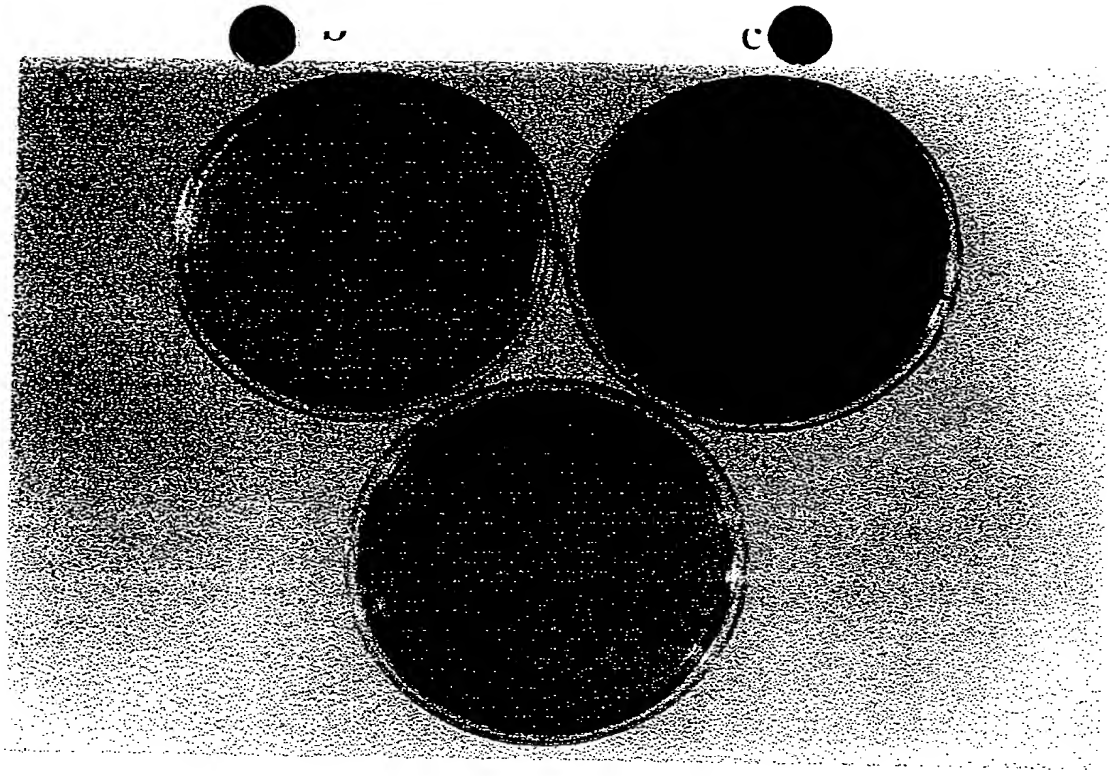


Figure 9 ^{1}H NMR of crude extracts of Pf5 (a) AN5 (b) from malt agar.



a

Figure 10a Potato Dextrose Agar with the indicator dye Bromocresol Purple added (plate a). This media with the parent strain AN5 streaked on it (plate b). The media has changed colour to yellow and is indication of a acidification of media. However this does not happen when the mutant strain AN5 - MN1 is streaked on the same media (plate c), infact the media goes more alkaline as indicated by the change in the colour of the media to purple.

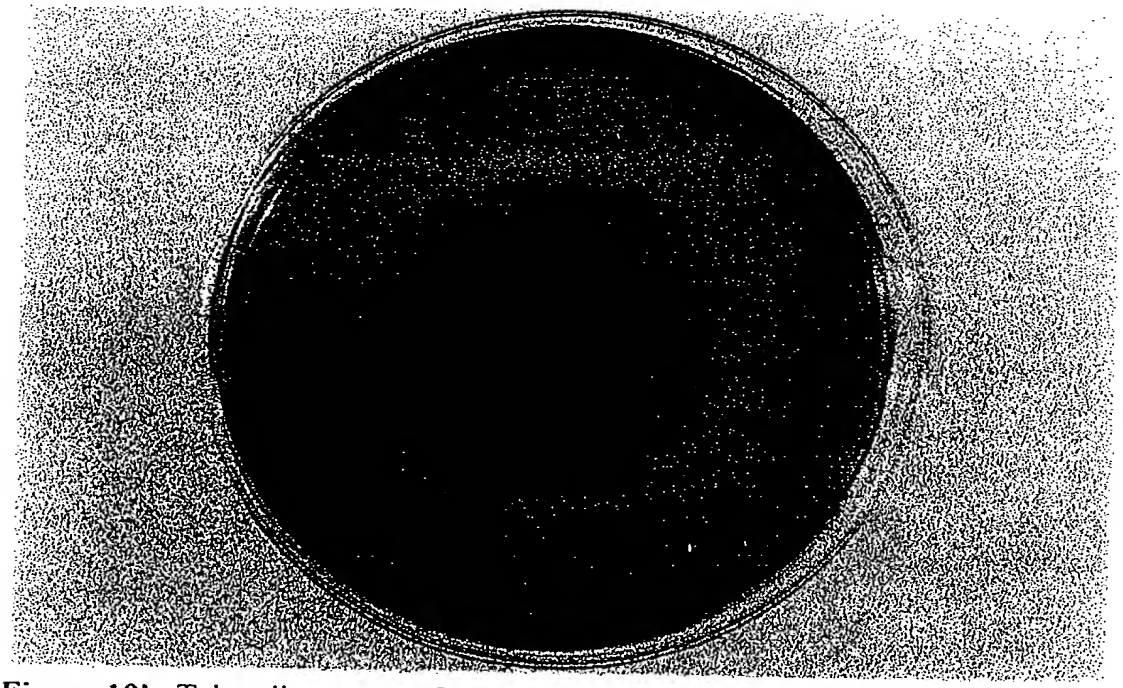


Figure 10b Take-all grown on Potato Dextrose Agar with the indicator dye Bromocresol Purple added. There is a purple hallow around the take-all as it grows suggesting it is releasing compounds which increase the pH of the media.

of Pseudomonas strain AN5 (pqqan5)

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pqqdkne  :  ------------tgtaatcatgcagaaaacgtccat  :  24
pqqdrn   :  ------------------------------------  :   -
pqqdanS  :  ------------------------------CAAAAC  :   6
pqqdaca  :  ttaaatgaataaagagcagtttgatttaaaccttgt  :  36

                    T  CG C  GG TA CG  T CA T  GA
pqqdkne  :  cgttgccttttcgtcgccggctacggactgccagctggga  :  60
pqqdrn   :  ----atgttttcgtcgccggctacggcatgcaaatttga  :  32
pqqdanS  :  CCCTCGCTGGCGCCCCGGCTATCGCTTCCAGTACGA  :  42
pqqdaca  :  gccaacatgggcgacaaggttacgcgttttcagttcga  :  72

                  C CA  A  G    GTG T  T TATCC GA
pqqdkne  :  agccgccccaggagagccatgtgatcctctatccggga  :  96
pqqdrn   :  aaaaacacaggactgccacgttgatttttgtatcctga  :  66
pqqdanS  :  ACCGGCGCAGAAAGGTCATCTCTTGCTCTATCCTGA  :  78
pqqdaca  :  gccagctcaaaatggttttgtgatttctatatcctga  :  108

                GG ATG   AA  T AA GA A  GC   G   AT
pqqdkne  :  gggtatggccgaaactcaatgagaccgccgcggcgat  :  132
pqqdrn   :  agggatggcgaaaactcaacgacagtgcgacgtttat  :  104
pqqdanS  :  ACGCATGCATCAAGCTCAACGACAGCGCGTGCGCTGAT  :  114
pqqdaca  :  aggcatgattaagttaaatgagagtgcagcggccaat  :  144

                      T GA GG    C       T  C
pqqdkne  :  cctagagctggttcgatggccggcgccgacgtccgcggc  :  168
pqqdrn   :  tttgcaactggttcgacggcgagcggacgattgccaa  :  140
pqqdanS  :  CGGCGGCCTGATTCGACGCTGAACGGGATGTCGCAGC  :  150
pqqdaca  :  cgggcaatatattgatgggcagcaaaatgtttctgc  :  180

               AT AT G    G T     A    TT    GA
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pqqdrn   :  tatcattgatgagctgaatgagcgcttccctgaagc  :  176
pqqdanS  :  CATCATCGGCGAGCTGGCCAAGCAGTTTCCCGACGT  :  186
pqqdaca  :  aattattgctcagttaaagcaaaaattggtgatat  :  216

                  G   T   T A GA  T     A T   T
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pqqdanS  :  GCCCGAACTCGGTGACGACATCGAGCAGTTCATGGA  :  222
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                  GC         A  A TGGAT
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pqqdrn   :  tcaggcccatgcacaaaagtggattaccttccgtga  :  248
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